Protein phosphorylation in mammalian sperm during capacitation

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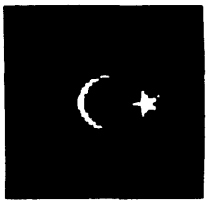
This thesis is dedicated to all Libyan martyrs who sacrificed their lives for others to live in freedom. We will never forget you



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Abstract

Sperm need to undergo a series of capacitation events in the female tract prior to fertilisation which are in part mediated by signalling events involving protein phosphorylation. This study investigated the dynamics of serine/threonine (S/T) protein phosphorylation in sperm incubated under capacitating (C) compared with non-capacitating (N) conditions using boar sperm as a model system.

For the first time, multiple bicarbonate-dependent S/T dephosphorylation events were observed in C compared with N mammalian sperm. Specifically, dephosphorylation of pp97, pp96, pp90, pp64 and pp55 was detected by using 2 phospho Akt substrate antibodies and a phospho kinase PKA substrate antibody. Dephosphorylation of pp105 was also detected using a phospho ATM/ATR substrate antibody. In contrast, no dephosphorylation was observed using a phospho PKC substrate antibody. Immunolocalisation revealed subtle changes in expression patterns as well as reduced phosphorylation in C sperm. dbcAMP/IBMX did not cause protein dephosphorylation in N sperm nor change the dephosphorylation previously observed in C sperm. However, it increased phosphorylation of p68, p51 and p29 in C sperm. Finally, the protein phosphatase inhibitor calyculin A prevented dephosphorylation of pp97, pp96, pp90, pp64, and pp55 but not pp105 indicating two pathways for dephosphorylation.

Subcellular fractionation revealed that pp97, pp96 and pp64 are head protein whereas pp90 and pp55 are tail proteins. Advanced proteomic analysis (GeLC-MS) identified 37 proteins, including AKAP4, AKAP3, CALI, HSPA1L and HSP70 as candidates for the dephosphorylated proteins. AKAP4 was excluded because it was localised to the tail. Two AKAP3 antibodies showed non-specific binding and a better quality antibody will be needed for further investigations. CALI was excluded because it was localised to the tail fraction. HSP70/72 and HSPA1L were strong candidates for pp64. However, immunoprecipitation of dephosphorylated proteins using phospho (S/T) PKA substrate Ab and HSPA1L Ab was unsuccessful and further work is now required to address this.

Publications and presentations

Publications (see appendix V for a copy)

Alnagar, F. A., Brennan, P. and Brewis, I.A (2010). Bicarbonate-dependent serine/threonine protein dephosphorylation in capacitating boar spermatozoa. *J Androl* **31**, 393-405.

Internal presentations

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Alnagar, F. A., Brewis, I.A and Brennan, P. (2008). Characterisation of serine/threonine and tyrosine protein phosphorylation in boar spermatozoa during capacitation (Poster presentation). Postgraduate Research Day. School of Medicine, Cardiff University, Cardiff. UK.

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Conference abstracts

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Abbreviations

1DE	One dimensional electrophoresis
2DE	Two dimensional electrophoresis
A	Acrosome
Ab	Antibody
ACN	Acetonitrile
Acr-2	Acrosin antibody
AKAP3/4	A kinase anchor protein 3
AnR	Annular ring
APM	Apical plasma membrane
ApR	Apical ridge
AR	Acrosome reaction
ATP	Adenosine triphosphate
BPS	Phosphate-buffered saline
BSA	Bovine serum albumin
CABYR	Calcium-binding tyrosine phosphorylation-regulated protein
CALI	Calicin
cAMP	Cyclic adenosine monophosphate
CASA	Computer assisted semen analysis
cBiMPS	Sp-dichloro-1-β-D-ribofuranosyl-benzimidazole-monophosphorothioate
CHCA	α-cyano-4-hydroxycinnamic acid
CTC	Chlortetracycline assay
DAPI	4, 6 dimidine-2-phenylindole dihydrochloride
dbcAMP	Dibutyryl-cAMP
DMSO	Dimethyle sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EqSs	Equatorial subsegment
ERK	Extracellular signal-regulated kinase
F	Flagellum
FITC	Fluorescein isothiocyanate
FS	Fibrous sheath
FSH	Follicle stimulating hormone

g	Relative centrifugal field
GeLC-MS	Gel liquid chromatogram-mass spectrometry
GnRH	Gonadotropin releasing hormone
GSK3	Glycogen synthase kinase-3
H	Head (of sperm cells)
HCO_3^-	Bicarbonate ion
HDL	High density lipoprotein
HSP	Heat shock protein
IAM	Inner acrosomal membrane
IBMX	3-isobutyl-1-methylxanthine
ICSI	Intracytoplasmic sperm injection
IIF	Indirect immunofluorescence
IP	Immunoprecipitation
IP3R	Inositol 1, 4, 5-trisphosphate receptors
IPG	Isoelectric focussing
IVF	*In vitro* fertilisation
LC	Liquid chromatography
LH	Luteinising hormone
MALDI TOF/TOF	Matrix assisted laser desorption ionisation time of flight-time of flight
MAPK	Mitogen activated protein kinase
MP	Midpiece
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
OAM	Outer acrosomal membrane
ODF	Outer dense fibres
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PE	Postequatorial region
PI3K	Phosphoinositide 3-kinase
PKA/B/C	Protein kinase A/B/C

PM	Plasma membrane
PNA	Peanut agglutinin
PP	Principal Piece
PP1/2A/2B	Protein phosphatase
p	protein
pp	phosphoprotein
PR	Posterior ring
PT	Perinuclear theca
PTK	Protein tyrosine kinase
PTP	Phosphotyrosine phosphatases
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene Fluoride
RI/II	Regulatory subunit I/II
RII	Regulatory subunit
ROS	Reactive oxygen species
Rp-cAMP	Rp-adenosine-3-5-monophosphate
RyR	Ryanodine receptor
S	Serine
sAC	Soluble adenylyl cyclase
SACY	Soluble adenylyl cyclase
SCX	Strong cation exchange
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	The mean and standard error of the mean
T	Tail (of sperm cells)
T	Threonine
TBS	Tris buffer solution
TFA	Trifluoroacetic acid
tmAC	Transmembrane adenylyl cyclase
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight- line velocity
v/v	Volume per volume
W	Whole sperm
w/v	Weight per volume

WHO	World Health Organisation
XIC	Extracted ion chromatogram
Y	Tyrosine
ZP	Zona pellucida

CHAPTER 1

Introduction

1.1 Mammalian fertilisation

Mammalian fertilisation is the union of sperm cells and eggs where the sperm cell fuses with the egg (oocyte) to create a zygote that is made up of the DNA from the parents (Evans and Florman, 2002) . However, in some cases natural mating does not lead to fertilisation and the infertile couple can benefit from *in vitro* fertilisation (IVF). Male factor infertility is primarily diagnosed by abnormal semen parameters, such as concentration, motility and morphology and is the single most commonly defined cause of infertility (Larsen et al., 2000). Such analysis of male fertility can only be used as guide to male factor infertility but does not represent definitive prediction of fertility. Therefore, understanding sperm function and different molecular pathways may help to develop simple, robust and effective tests of sperm function and semen quality for fertilisation.

Major progress has been achieved in spermatology in the last two decades, including the successful transplantation of fresh and cryopreserved testicular tissues (Avarbock et al., 1996; Brinster and Avarbock, 1994), the reported production of spermatogenic cells from stem cells (Geijsen et al., 2004; Hayashi et al., 2011) and intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992). However, the underlying biochemical, molecular and genetic causes of male infertility is still poorly understood.

Molecular mechanisms in sperm cells prior to and during fertilisation are also far from being completely understood. Therefore, further fundamental research is needed to elucidate these processes to better understand the important biological events of the fertilisation process. This knowledge might be applied to improve assisted

conception. In addition, more explanation of the fertilisation background could help to discover new methods to control reproduction beyond hormone contraceptive drugs, which have certain side effects (Aitken et al., 2008; Li and Anderson, 2010; Lopez et al., 2011).

Prior to fertilisation sperm cells must undergo a number of changes to be able to reach and fertilise the eggs. These changes usually occur naturally *in vivo* (in the female tract) and are termed capacitation. This may also be induced *in vitro* in a defined media (Visconti et al., 2002). Following capacitation, sperm cells must then bind to the zona pellucida (ZP) which is the extracellular matrix of the egg (Kim et al., 2008; Wassarman and Litscher, 2008). Zona binding induces the release of the acrosomal contents of the sperm which is called the acrosome reaction (AR) (Florman et al., 2008; Florman et al., 1989). The fertilising sperm cell then fuses with the egg (Figure 1.2). Following fertilisation and the formation of the zygote, further sperm are not able to bind the egg to avoid polyspermy (Wassarman et al., 2001).

After a long time and many failures to achieve successful *in vitro* fertilisation (IVF), the discovery of capacitation played a role that made it possible to produce the first successful 'test-tube' baby (Steptoe and Edwards, 1978). Although capacitation was discovered sixty years ago, the molecular mechanism and the exact sequence of events are still not fully understood. An improved knowledge of capacitation mechanisms is important to better understand pre-fertilisation events, including zona binding and the AR.

1.2 Mammalian spermatogenesis

Fully mature sperm cells capable of capacitation and fertilisation are produced in the testis. Spermatogenesis is a dynamic process to synthesise sperm from stem cells in the testis and starts at puberty (Sutovsky and Manandhar, 2006). Spermatogenesis takes place in the seminiferous epithelium lining the seminiferous tubule. The seminiferous epithelium is comprised solely of spermatogenic cells and Sertoli cells. There are three different germ cell types that are constitutively present in the testis of the adult male include spermatogonia to spermatocytes to spermatids. Spermatogenesis can be divided into two parts, namely the diploid and the haploid phase. During the diploid phase two meiotic divisions occur resulting in round haploid spermatids. Moreover, during the haploid phase, which is called spermiogenesis, the morphological and functional characteristics of the sperm are determined (Sutovsky and Manandhar, 2006).

Sperm released from the testis are unable to fertilises egg and they must undergo a period of maturation as they pass through the epididymis (Cooper, 1990). Moreover, during transit through the proximal epididymidis various changes to sperm culminate in their acquisition of progressive motility and the ability to recognise, bind to the ZP and fuse with the oolemma (Cooper, 2007; Moore, 1990; Moore and Akhondi, 1996). After their maturation epididymal sperm may be stored for a variable period in the distal epididymidis and vas deferens before ejaculation (Yanagimachi, 1994).

1.3 The structure of mammalian sperm

Mammalian sperm are highly polarised cells with the haploid genome tightly packaged in the head and a flagellum that generates the motility needed to deliver the

sperm to the egg. Mature sperm cells are capable of acquiring progressive motility in the epididymis and fertilising potential in the oviduct (Yanagimachi, 1994). Sperm are composed of a head and flagellum joined by the midpiece and covered by plasma membrane (Figure 1.1). The sperm head consists of the nucleus which is composed of DNA linked and compacted by histones and replaced by protamines during spermatogenesis (Brewer et al., 2002; Miller et al., 2010). The anterior part of the head is capped by the acrosome which is a large vesicle containing the hydrolytic enzymes. These enzymes are necessary for digesting the coat of the ZP during fertilisation (Yoshinaga and Toshimori, 2003). The acrosomal membrane can be divided into the inner (IAM) and outer acrosomal membrane (OAM) [for review see (Eddy and O'Brien 1994)]. Furthermore, the acrosomal plasma membrane in the pre-equatorial region is separated from the post-equatorial membrane by the equatorial region which has a species-specific size and shape (Sutovsky and Manandhar, 2006; Yanagimachi, 1994). The plasma membrane of the pre-equatorial region and the apical region in the anterior sperm head are known to be involved in zona binding, the acrosome reaction and fusion (Gadella et al., 2008).

The sperm head cytoskeleton is called the perinuclear theca which covers the nucleus and consists of many proteins (Bellve et al., 1992). The sperm head cytoskeleton gives the sperm head the final structural shape and supports its function for egg penetration and fertilisation (Yanagimachi, 1994). Although most sperm heads in mammals are circular in cross section, the eutherian sperm head is usually flattened in one plane, allowing it to oscillate in that plane during zona penetration (Bedford, 1994). On the other hand, rodent sperm heads are hook-shaped (falciform).

The sperm flagellum is an important and complex structure that provides the sperm with hyperactivated motility (asymmetrical flagellar bending motility) to fertilise the egg (Suarez, 2008; Suarez and Ho, 2003; Suarez et al., 1993). The motor of the flagellum is the axoneme which spans the entire length of the tail and is composed of a central pair of microtubules surrounded by nine outer doublets of microtubules and is located at the centre of the flagellum throughout its full length. The flagellum of mammalian sperm has four distinct segments: the connecting piece adjacent to the head, the midpiece defined by a tightly packed helical array of mitochondria surrounding the cytoskeletal structures of the flagellum, the principal piece and the short Endpiece (Yanagimachi, 1994). The connecting piece consists of centriolar and pericentriolar material. Both the proximal centriole and the remnants of the distal centriole are made of tubulin (Sutovsky and Manandhar, 2006).

The axoneme is surrounded in the midpiece and principal piece by the outer dense fibres (ODF) which are the cytoskeletal structures novel to the sperm flagellum in higher vertebrates. The connecting piece of the head membrane is separated from the midpiece by the posterior ring and this is connected to the tail by the annular ring. The principal piece makes up about three-quarters of the length of the flagellum and is defined by the fibrous sheath (FS) which underlies the plasma membrane and surrounds the outer dense fibres. The terminal segment of the flagellum is the end piece and lacks the structures associated with the axoneme (Eddy et al., 2003). The cytoskeletal components of the flagellum are responsible for generating motion. The interaction between ATP and ATPases on the microtubule doublets promotes their sliding and causes the flagellum to bend (Fouquet and Kann, 1994). Although the structural features of the flagellum have been well described, little is known about how

changes in flagellar function are regulated and specific roles of its unique cytoskeletal components.

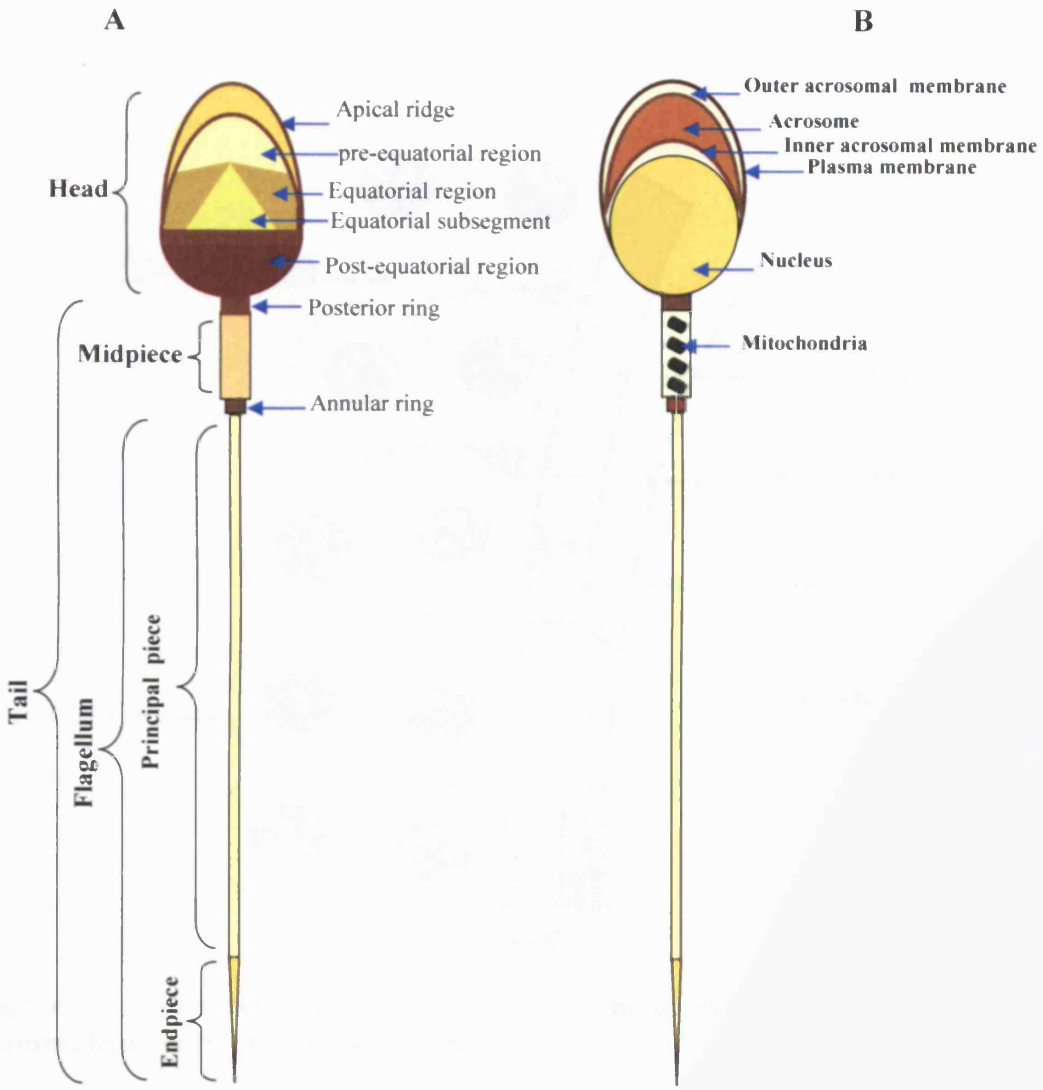

Figure 1.1: Schematic representation of boar sperm.

(A) A surface view of the sperm head, midpiece and flagellum. The head can be subdivided into four distinct domains: the apical ridge, pre-equatorial, equatorial, equatorial subsegment (EqSs) and post-equatorial regions. The sperm flagellum consists of the principal piece and the endpiece and enables sperm motility. **(B)** Cross section of a sperm cell showing the sperm head and flagellum covered by plasma membrane (PM). The head contains the nucleus and the anterior part of the head is covered by the acrosome which is a large, secretory vesicle containing hydrolytic enzymes. the inner acrosomal membrane (IAM) covers the apical nuclear envelope, and the outer acrosomal membrane (OAM) is close to the plasma membrane in the head region. The midpiece contains mitochondria which provides the sperm cells with energy. This figure was adapted from Flesch et al. (2000).

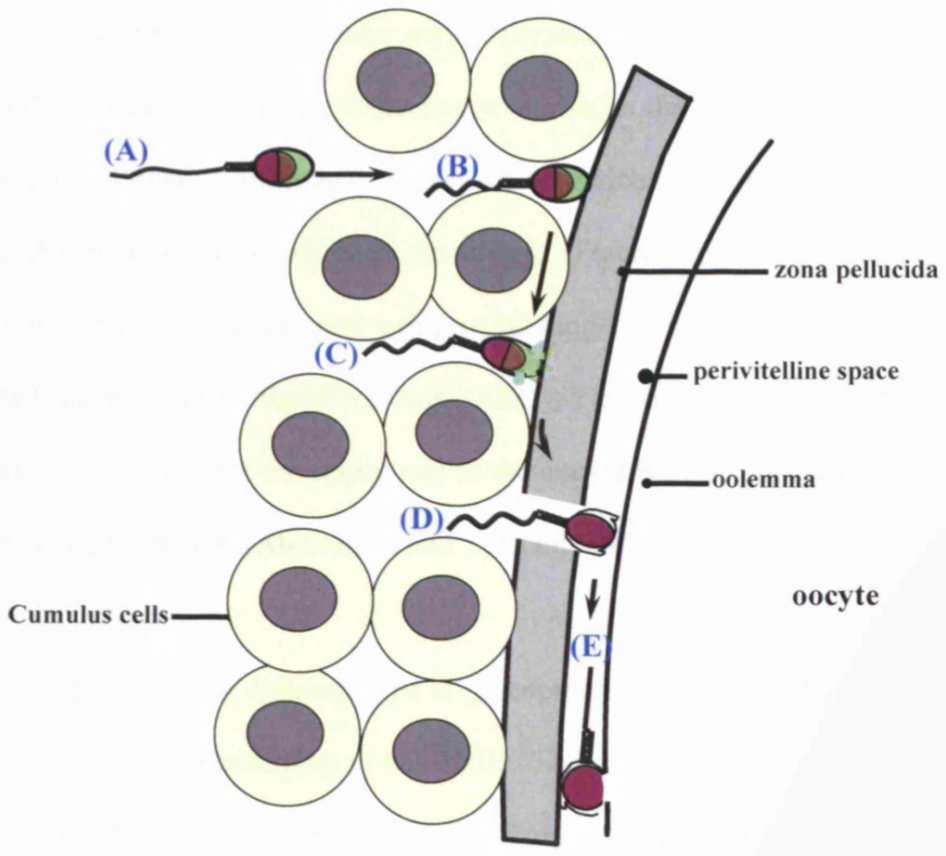

Figure 1.2: Schematic representation of the main events occurring in mammalian sperm during fertilisation.

(A) Freshly ejaculated sperm are activated in female tract during capacitation. **(B)** Capacitated sperm displaying hyperactived motility, penetrate the cumulus cells and bind to the zona pellucida (ZP). **(C)** Sperm binding to ZP induces the acrosome reaction (AR) in which the outer acrosomal membrane fuses with the plasma membrane, releasing the acrosomal contents. **(D)** The acrosome contains hydrolytical enzymes which degrade the ZP and enable sperm to penetrate the ZP and enter the perivitelline space. **(E)** The sperm cell binds via the equatorial region to the oolemma and, subsequently fuses with the oocyte. This figure was adapted from Flesch et al. (2000).

1.4 Sperm-zona binding

Fully mature mammalian oocytes that are ready for fertilisation are each surrounded by a thick vitelline envelope called the ZP that is surrounded by numerous follicular cells embedded in a cellular matrix (hyaluronic acid polymers) (Wassarman et al., 2001). Together, these are collectively known as the cumulus-oocyte complex (Yanagimachi, 1994). Capacitated sperm cells which become hyperactived first penetrate the cumulus mass to reach the oocyte (Figure 1.2). An important step in fertilisation is the recognition and primary binding of the sperm to the ZP. Only capacitated sperm bind to the ZP (Ahuja, 1985; Yanagimachi, 1994). This binding event serves not only to fix the sperm cell to the oocyte but also induces the signalling cascade leading to the AR (Abou-haila and Tulsiani, 2009).

The ZP consists of different type of glycoproteins (Bleil and Wassarman, 1980; Lefievre et al., 2004; Wassarman et al., 2001). These proteins are synthesised by the oocyte and post-translationaly modified by glycosylation at serine/threonine (S/T) and at aspargine residues (Wassarman, 1988). The size, rigidity and the thickness of the ZP varies from species to species. It varies in thickness form <2 μm in marsupials to 27 μm in cows (Maresh and Dunbar, 1987). Various ZP glycoproteins constitute the matrix have been characterised in several species. In the mouse, the ZP is composed of three families of glycoproteins nominated ZP1, ZP2 and ZP3 with molecular weights of 180, 120 and 83 kDa, respectively based on SDS-PAGE (Bleil and Wassarman, 1980). Characterisation of human ZP glycoproteins by various groups has revealed heterogeneity in the mobility on SDS-PAGE (Bauskin et al., 1999; Gupta et al., 1998). In contrast to the mouse, human ZP is composed of ZP1, ZP2, ZP3 and ZP4 (Conner et al., 2005; Lefievre et al., 2004). Porcine ZP consists of three glycoproteins referred to

as ZP2, ZPB and ZP3 using nomenclature according to (Hughes and Barratt, 1999). In pig, ZPB, ZP3 are thought to be directly involved in sperm binding (Yurewicz et al., 1983; Yurewicz et al., 1998).

The sperm cell binds to the ZP by zona receptors localised on the apical plasma membrane. One candidate is a 95kDa protein which has been reported in several mammalian species as a putative ZP receptor (Leyton and Saling, 1989). The protein is phosphorylated on tyrosine residues (Brewis et al., 1998; Visconti et al., 1995b) and this phosphorylation increases during *in vitro* capacitation (Brewis et al., 1998; Leyton and Saling, 1989). However, there has been much controversy about the 95 kDa which has not been definitively identified (Brewis and Wong, 1999). Sperm ZP binding has also been studied by the isolation of the apical plasma membrane (APM) from sperm cells using a nitrogen cavitation method (Flesch et al., 1998). Isolation of the apical plasma membrane from the intact sperm facilitated the identification of multiple sperm proteins binding to the ZP using two dimensional electrophoresis (2DE) and protein identification by tandem mass spectrometry (van Gestel et al., 2007). Overall current thinking is that multiple proteins are involved in ZP binding but the process is still far from being fully understood.

1.5 Acrosome reaction

The acrosome reaction (AR) is an exocytotic process initiated immediately after primary binding of a capacitated sperm cell to the zona pellucida of the oocyte (Figure 1.2) (Buffone et al., 2008; Yanagimachi, 1994). The AR consists of multiple fusions between the outer acrosomal membrane and the overlaying plasma membrane leading to the secretion of hydrolytic and proteolytic enzymes in order to hydrolyse and dissolve the ZP to allow sperm to penetrate to the perivitelline space (Figure 1.2)

(Bedford, 2008). The dogma states that capacitated sperm cells are physiologically able to undergo AR in response to physiological stimulators (Patrat et al., 2000). Therefore, capacitation and AR are sequentially and functionally linked such that several of the effectors involved in mediating intracellular signalling activated by the AR start to be turned on during capacitation (Baldi et al., 2000). Therefore, the AR should not proceed prior to ZP binding because the part of the enzymatic machinery of this organelle required for successful zona penetration will be lost. However, the AR is an irreversible process and prematurely acrosome reacted sperm are considered to be incompetent to fertilise the oocyte (Florman et al., 2008).

Although many molecular mechanisms have been demonstrated to be active during the AR, it is still unclear whether these mechanisms are related to fertilisation or just associated with the AR; for review see (Baldi et al., 2000). The initiation of the AR is dependent on a massive increase in intracellular Ca^{2+} levels in the sperm cell. Ca^{2+} influx is an absolute requirement for the physiological AR in sperm (Jimenez-Gonzalez et al., 2006; Publicover and Barratt, 1999). The AR can be induced in response to progesterone (Guerrero and Darszon, 1989) and can also be induced when sperm cells are exposed to progesterone and then to ZP (Roldan et al., 1994). Moreover, sperm cells that underwent stimulus-induced AR (progesterone or calcium ionophore A23187) are more viable and motile than sperm that underwent premature (spontaneous) AR (Harper et al., 2008).

1.6 Sperm capacitation

As previously introduced freshly ejaculated sperm do not possess the ability to fertilise an oocyte. Therefore, sperm must undergo a series of events in the female

reproductive tract that enables them to fertilise the oocytes (Yanagimachi, 1994) and collectively these are known as capacitation (Austin, 1952; Chang, 1951).

1.6.1 Capacitation in the female oviduct

Mammalian capacitation naturally takes place in the female genital tract. Semen is deposited in the anterior vagina during coitus in certain species, such as cow, sheep, rabbit and primates. In other species, including, pig, horse, dog and rodents semen enters the uterus directly (Hunter, 1980). In mammals, oocytes are usually fertilised within hours of ovulation (Austin, 1957). However, in some species, sperm may be inseminated days (pigs, cattle and horses) or months (some bat species) before the arrival of the oocyte. In humans, there is evidence that fertilisation takes place when intercourse occurs up to five days prior to ovulation (Wilcox et al., 1995).

1.6.2 *In vitro* capacitation

The development of culture systems that support capacitation and fertilisation *in vitro* has made it possible to study in detail the requirements for the acquisition of fertilising ability. Early attempts to induce capacitation *in vitro* used various biological fluids, such as blood serum (Miyamoto and Chang, 1973; Yanagimachi, 1970) follicular fluids and oviduct fluids (Barros, 1968). However, it was difficult to determine the main component responsible for inducing capacitation using these fluids. Moreover, capacitation can also be induced by incubation in chemically defined media without such biological fluids (for example commonly used Tyrode's and Krebs-Ringer's media). Although minor variations exist between these media, depending on the mammalian species, most contain bicarbonate (HCO_3^-), calcium (Ca^{2+}), low potassium (K^+), a cholesterol acceptor (serum albumin) and energy sources (such as

glucose, lactate and pyruvate) (Visconti and Kopf, 1998). The mechanism by which most of these compounds promote capacitation is not fully understood at the molecular level. However, molecular changes associated with capacitation for example tyrosine phosphorylation, have been widely reported (Flesch et al., 2001a; Jha and Shivaji, 2002a; Tardif et al., 2001; Visconti and Kopf, 1998; Visconti et al., 1995a).

1.7 Hyperactivated motility

Hyperactivated motility is a high-amplitude flagellar bending and was first described by Yanagimachi when hamster sperm were incubated in follicular fluid or serum (Yanagimachi, 1969; Yanagimachi, 1970). At first this motility was described as 'activation' but later the term was changed to 'hyperactivation' to distinguish it from the initial motility in seminal plasma (Yanagimachi, 1984). This hyperactivated motility has been observed in many species, such as rabbits (Overstreet and Cooper, 1978; Young and Bodt, 1994), mice (Fraser, 1977; Neill and Olds-Clarke, 1987), pigs (Suarez et al., 1992) and humans (Mortimer and Swan, 1995). Sperm hyperactivated motility varies among species but it is an important event of capacitation. It is associated with an increased velocity, a decreased linearity, increased amplitude of lateral head displacement, and whiplash movements of the flagellum (de Lamirande et al., 1997). Initial studies observed hyperactivated motility during capacitation *in vitro* and in sperm flushed from the oviduct near the time of fertilisation and it may be important for transport through the female tract and facilitate penetration of the ZP (Stauss et al., 1995; Yanagimachi, 1994).

1.8 Media components and the molecular basis of sperm capacitation

The extracellular environment plays a crucial role in promoting or inhibiting functional changes in mammalian sperm (Fraser, 2010). Caudal epididymidial and ejaculated sperm can be incubated under a variety of conditions in media that mimics the electrolyte composition of the oviductal fluids. The putative mechanism of action of these components is still poorly understood (Figure 1.3). *In vitro* capacitation occurs over several hours and current evidence indicates that it is mediated by signalling events involving protein phosphorylation due to various kinases and transduction pathways (Harayama and Nakamura, 2008; Harayama et al., 2004a; Tardif et al., 2001; Visconti et al., 1995b). Although there are variations in the media used for *in vitro* capacitation most contain bicarbonate, calcium and BSA (Visconti and Kopf, 1998).

1.8.1 Bicarbonate

It is well established that capacitation is a bicarbonate dependent process (Boatman and Robbins, 1991; Gadella and Harrison, 2000; Lee and Storey, 1986; Visconti et al., 2002). Many studies have demonstrated that bicarbonate is one of the most important constituent in both *in vivo* and *in vitro* sperm capacitation and plays a major role in the induction of capacitation *in vitro* (Lee and Storey, 1986; Boatman and Robbins, 1991; Visconti et al., 1995). Bicarbonate is particularly important for inducing capacitation in boar sperm (Flesch and Gadella, 2000; Harrison, 1996; Okamura et al., 1985). Considering bicarbonate as an essential component of *in vitro* capacitation, it mimics two important aspects of capacitation *in vivo*. Physiologically, sperm migrate from a low bicarbonate concentration in seminal plasma (Asari et al., 1996) to the female reproductive tract (lower portion of the isthmus of the oviduct and probably in the uterus) which has a high bicarbonate concentration (about 20 mM)

(Rodriguez-Martinez et al., 2001; Tienthai et al., 2004). Interestingly, the low concentration of bicarbonate in the epididymis maintains sperm in an environment that does not support capacitation until sperm reach the higher concentrations of bicarbonate in the female oviduct and undergo capacitation. This effect of bicarbonate on sperm is supported by evidence regarding defective bicarbonate secretion in the female genital tract which causes impaired sperm capacitation and lower female fertility (Wang et al., 2003).

Bicarbonate plays an important regulatory role in promoting capacitation *in vitro* in all species studied so far and takes several hours and varies considerably in different species (Flesch et al., 2001b; Gadella and Harrison, 2000; Harrison, 1996; Harrison et al., 1996; Lee and Storey, 1986; Yanagimachi, 1994). Sperm are usually incubated *in vitro* usually at the temperature of the female tract of a particular species (37-39 °C). Moreover, sperm incubation usually takes place in 5% CO_2 in air to enable bicarbonate diffuse into sperm.

The addition of bicarbonate to the capacitation media is important for capacitation (Harrison et al., 1996) and fertilisation in the pig (Suzuki et al., 1994a; Suzuki et al., 1994b). It was reported that bicarbonate causes a lateral redistribution of cholesterol in the plasma membrane of pig sperm, which in turn facilitates cholesterol extraction by serum albumin (Flesch et al., 2001b). This lipid disordering causes changes in membrane fluidity (Gadella and Harrison, 2002; Gadella and Van Gestel, 2004). Membrane fluidity could also be influenced by the increase in temperature that occurs in the isthmus of the oviduct at the time of ovulation in boar sperm (Hunter and Nichol, 1986).

It was not clear exactly how bicarbonate could enter sperm but it was found in mouse sperm that a Na^+/HCO_3^- cotransporter appeared to be involved (Demarco et al., 2003; Romero and Boron, 1999). However, two possible mechanisms for HCO_3^- entry into the sperm cells were proposed; by anion transport of CO_2 across the cell membrane via a carrier or by direct diffusion of CO_2 through the membrane with subsequent hydration to bicarbonate (Carlson et al., 2007). At the biochemical level, the transmembrane movement of HCO_3^- anions into sperm raises the intracellular pH, stimulates respiratory activity and facilitates the opening of Ca^{2+} channels. This directly activates the special form of soluble adenylyl cyclase (SACY) and promotes the generation of cyclic adenosine monophosphate (cAMP) (Okamura et al., 1985). SACY activity increases during capacitation and exogenous analogues of cAMP and/or inhibitors of cyclic nucleotide phosphodiesterases have been shown to accelerate capacitation *in vitro* and is reviewed by (Fraser and Monks, 1990). Importantly, bicarbonate has also been reported to cause rapid changes in the sperm plasma membrane architecture (Gadella and Harrison, 2002; Harrison, 1996; Harrison and Miller, 2000).

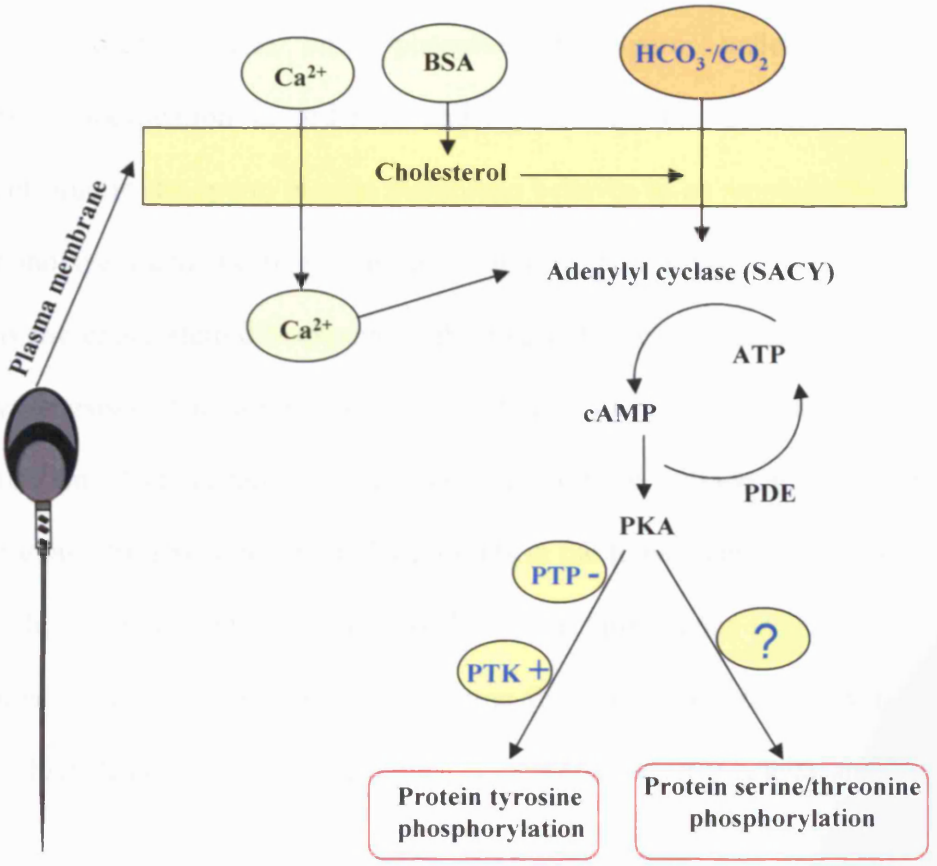

Figure 1.3: Proposed major of signalling pathways in mammalian sperm capacitation.

Bicarbonate (HCO_3^-) enters the cell via a Na^+/HCO_3^- co-transporter and binds to soluble adenylyl cyclase (SACY). This binding activates the SACY and causes increased production of cAMP that in turn activates protein kinase A (PKA). The role of cholesterol efflux in the activation of PKA is unclear. Cholesterol efflux may induce increased bicarbonate entry or may affect SACY. PKA induces tyrosine phosphorylation of several substrates via the activation of protein tyrosine kinase (PTK) or inhibition of protein tyrosine phosphatases (PTP). PKA also activates serine/ threonine (S/T) phosphorylation but the actual mechanism is not clear. Bovine serum albumin (BSA) activates the redistribution of cholesterol in the plasma membrane. The removal of this sterol could account for the membrane fluidity changes during capacitation. The role of Ca^{2+} in initiating and/or regulating capacitation is controversial. This figure was adapted from Visconti et al. (2002).

1.8.2 Efflux of plasma membrane cholesterol

A freshly ejaculated sperm has high amount of lipids in the plasma membrane including cholesterol and other phospholipids. Sperm undergo major plasma membrane modification in order to undergo fertilisation. Cholesterol is the most abundant lipid in the sperm plasma membrane behaves as an amphipathic molecule in both monolayers and the free hydroxyl group in cholesterol is the hydrophilic part, whereas the entire steroid backbone is the hydrophobic part (Gadella et al., 2008). It was hypothesised that during sperm capacitation, bicarbonate first induces a lateral redistribution of cholesterol and facilitates cholesterol extraction by serum albumin (Flesch et al., 2001b) which naturally presents in the female genital tract (Benoff et al., 1993a; Benoff et al., 1993b; Go and Wolf, 1985). This change in lipids of the plasma membrane leads to an increase in membrane fluidity and consequently increases ion fluxes which leads to plasma membrane hyperpolarisation (Gadella and Van Gestel, 2004).

It was demonstrated that incubation of mouse sperm in medium containing bovine serum albumin (BSA) results in a release of cholesterol from the sperm plasma membrane to the medium; release of this sterol does not occur in medium devoid of BSA (Visconti et al., 1999b). BSA is believed to be required for capacitation as a consequence of its ability to serve as a cholesterol-binding molecule but it is still not known if the only action of BSA is through the removal of cholesterol (Yanagimachi, 1994). Although the requirement of BSA for in vitro capacitation is necessary in some species, in pig sperm BSA may be needed for fertilisation but not for capacitation (Suzuki et al., 1994a). In this context, albumin had no effect on cholesterol of mammalian sperm in the absence of bicarbonate (Flesch et al., 2001b).

The effect of cholesterol efflux on signal transduction pathways during capacitation is still incompletely understood. It is possible that prior to capacitation cholesterol assembles in the plasma membrane domains or lipid rafts which act to bring the plasma membrane proteins together. These membrane proteins in the lipid rafts are very important players in fertilisation especially that they function as receptors to bind the ZP and are also involved in the egg fusion process (Brewis et al., 2005). One of these proteins is caveolin-1 which is encoded by caveolin-1 gene and present in the plasma membrane overlying the acrosomal and flagellum of mouse and guinea pig sperm (Travis et al., 2001). It was believed that removal of cholesterol from the plasma membrane is upstream of multiple signalling events and plays a role in modulating signalling pathways in sperm cells (Visconti et al., 1995a; Visconti et al., 1995b). HCO_3^- induces PKA-dependent changes in the lipid architecture of the sperm plasma membrane (Harrison and Miller, 2000), due to phospholipid scrambling (Gadella and Harrison, 2000).

The role of cholesterol in the activation of sperm cells during capacitation has been studied using cyclodextrin which is an agent that extracts cholesterol from membranes. It has been demonstrated that sperm cells incubated in the absence of bicarbonate but with cyclodextrin had markedly activated PKA (van Gestel et al., 2005; Visconti et al., 1999c) and enhanced tyrosine phosphorylation levels (Cross, 1999; Visconti et al., 1999c). Additionally, serum albumin can be also substituted with high density lipoproteins (HDL) in the *in vitro* capacitation media which binds the cholesterol in the sperm plasma membrane and induce capacitation (Therien et al., 1997).

Membrane cholesterol organisation was observed with filipin which complexes cholesterol into clusters that can be visualised using freeze fracture techniques and electron microscopy at the level of the sperm surface. Filipin labeled the entire sperm surface of non-stimulated and non-responding cells, but labeled only the apical surface area of bicarbonate-responding cells (Flesch et al., 2001b). An increase of membrane fluidity occurs during capacitation and caused by an altered bilayer distribution of phospholipids (Gadella and Harrison, 2000). The lipid change is detected through an increased ability to bind merocyanine (a lipophilic fluorochrome) whose affinity for a given plasma membrane leaflet depends on the degree of lipid structural disorder in the leaflet (Harrison and Miller, 2000). The precise molecular mechanism signaled by increased merocyanine binding remains uncertain.

1.8.3 Calcium (Ca^{2+})

In mammalian sperm, calcium plays a pivotal role in many fertilisation processes including capacitation, hyperactivation, the AR and egg activation (Florman et al., 1989; Fraser, 1977; Ho and Suarez, 2001a; Yanagimachi and Usui, 1974) and occurrence of sperm egg fusion. Ca^{2+} is required for both capacitation and acrosome reaction but the amount required for each may differ markedly. Relatively little Ca^{2+}, for example low micromolar concentrations is required for capacitation in at least some species (e.g. guinea pig, mouse), but millimolar concentrations are required for maximal hyperactivation, acrosome loss and fertilization in all species evaluated, for review see (Fraser, 1995).

Particular attention has been paid to the ionic composition of the culture media by varying the concentration and the temporal availability of individual ions. It has

been possible to determine which ions are required specifically for *in vitro* capacitation and for the AR. Of these ions, the most important appears to be Ca^{2+}. Extracellular calcium is one of the main constituents for the completion of capacitation *in vitro* (Yanagimachi, 1994). However, the role of Ca^{2+} in initiating and/or regulating capacitation is currently controversial (Visconti et al., 2002).

An increase of intracellular Ca^{2+} during sperm capacitation was reported in many species (Yanagimachi, 1994). Elevation of cytoplasmic Ca^{2+} concentration can occur by entry of Ca^{2+} into cells through the plasma membrane or release of Ca^{2+} from membrane-bounded internal stores. The rise in Ca^{2+} during capacitation would also produce a stimulation of adenylyl cyclase. Consistent with these observations, recent studies on capacitation *in vitro* of mouse sperm have indicated a time-dependent increase in protein tyrosine phosphorylation during capacitation (Visconti et al., 1995a). Ca^{2+} has induced tyrosine phosphorylation of mouse sperm during capacitation. On the other hand, Ca^{2+} inhibited protein tyrosine phosphorylation in human sperm during capacitation within 2 h *in vitro* (Carrera et al., 1996; Luconi et al., 1996).

Ca^{2+} has been shown to be crucial for the initiation and maintenance of hyperactivated motility in hamster sperm (Suarez et al., 1993), and that cytoplasmic Ca^{2+} concentration within the flagellum per activated sperm is increased (Suarez and Dai, 1995; Suarez et al., 1993). The crucial site for the action of Ca^{2+} is the axoneme in the core of the flagellum (Lindemann et al., 1991). Thus, Ca^{2+} is a key regulator of hyperactivation, acts directly on the axoneme. Although there are two main sources of Ca^{2+} extracellular which brought through plasma membrane channels and Ca^{2+} stored

in organelles, the predominant source of Ca^{2+} for hyperactivation is the extracellular formed by proteins in the CatSper family (Kirichok et al., 2006). The CatSper proteins are localised to the sperm flagellum (Carlson et al., 2005). Null mutant mice for CatSper are infertile due to a failure for sperm to become hyperactivated (Ren et al., 2001).

1.8.4 Sodium (Na^+) and potassium (K^+) ions and plasma membrane hyperpolarisation

Sperm maintain an internal ion concentration different from that in the extracellular medium in normal conditions. The requirements for sodium and potassium ions in sperm capacitation have not been widely studied. It was found that guinea pig sperm needs very low concentrations of Na^+ (Hyne et al., 1984). In bull and mouse sperm, increased permeability of K^+ ions was associated with capacitation and would lead to sperm membrane hyperpolarisation (Zeng et al., 1995).

The difference *in vitro* between intracellular and extracellular ion concentrations is determined by the relative permeability of the sperm plasma membrane to the ions involved in the capacitation media. Sperm under non capacitating conditions have high Na^+ permeability and low K^+ which is called depolarisation whereas Na^+ decreased during sperm capacitation and the increased permeability of K^+ occurs and lead to hyperpolarisation (Hernandez-Gonzalez et al., 2006). These observations were supported the studies on Na^+/K^+ -ATPase inhibitors such as ouabain and amiloride (Hyne, 1984; Hyne et al., 1984). The Na^+/K^+ -ATPase acts as a sodium pump for the plasma membrane of animal cells including sperm (Thundathil et al., 2006).

Mammalian sperm capacitation is accompanied by the hyperpolarisation of the sperm plasma membrane (Zeng et al., 1995). Hyperpolarisation is observed as an increase in the intracellular negative charges when compared with the extracellular environment. Although it is not clear how sperm plasma membrane potential is regulated during capacitation, it appears that membrane hyperpolarisation may be partially because of an enhanced K^+ permeability as a result of a decrease in inhibitory modulation of K^+ channels (Zeng et al., 1995). It was suggested that the possible role of the sperm plasma membrane hyperpolarisation during capacitation, may be to regulate the ability of sperm to increase intracellular Ca^{2+} during acrosome reaction (Florman et al., 1998).

Hyperpolarisation associated with capacitation is regulated by the components of the capacitation media. It was been reported that incubation of mouse sperm in capacitation media devoid of BSA and bicarbonate, does not show the changes in plasma membrane potential (Demarco et al., 2003). This observation suggested that bicarbonate and cholesterol efflux in the capacitation media may play a direct and indirect function leading to the hyperpolarisation of the sperm plasma membrane. In addition, it was demonstrated recently that inward K^+ channels are expressed in mouse sperm and proposed that these channels may be responsible for the capacitation-associated membrane hyperpolarisation (Munoz-Garay et al., 2001).

1.8.5 Adenylyl cyclases during capacitation

SACY is the enzyme responsible for the intracellular production of cAMP which regulates a number of biological processes in various cell types (Okamura et al., 1985). There are two types of adenylyl cyclases that are responsible for cAMP

synthesis in eukaryotes. First is the transmembrane adenylyl cyclases (tmAC) which is regulated by G protein associated receptors and the second is the SACY which is insensitive to G protein and active in the presence of Mn+ and bicarbonate (Buck et al., 1999; Esposito et al., 2004).

Mammalian male germ cells contain an atypical adenylyl cyclase (Braun et al., 1977) now identified as SACY (formerly known as sAC for "soluble Adenylyl Cyclase")(Buck et al., 1999). SACY is directly stimulated by bicarbonate ions and appears to be the predominant form of adenylyl cyclase in mammalian sperm (Chen et al., 2000). The direct activation by bicarbonate provides a mechanism for generating the cAMP required to complete the bicarbonate-induced processes necessary for fertilisation, including hyperactivated motility, capacitation, and the acrosome reaction [for review see (Salicioni et al., 2007)]. Immunolocalisation studies reveal SACY is also abundantly expressed in other tissues which respond to bicarbonate or carbon dioxide levels suggesting it may function as a general bicarbonate/CO_2 sensor throughout the body (Baxendale and Fraser, 2003).

As a consequence of SACY activation by bicarbonate, intracellular levels of cAMP increase and activate protein kinase A (PKA) which phosphorylates many proteins that initiate several signal pathways in mammalian sperm (Visconti and Kopf, 1998; Visconti et al., 1995a). The importance of SACY involved in sperm capacitation was evidenced by the finding that SACY null mice sperm cells are morphologically normal but immotile and infertile. Interestingly, this sperm motility defect due to SACY deficiency was rescued by adding cAMP analog but it was insufficient to induce the other aspects of capacitation and consequently remain unable to fertilise the

eggs (Esposito et al., 2004). Furthermore, a study using pharmacologically developed SACY inhibitor was able to inhibit SACY and showed that capacitation events including the induction of tyrosine phosphorylation and motility in sperm is SACY-dependent (Hess et al., 2005).

1.8.6 Cyclic adenosine monophosphate (cAMP)/PKA pathway

cAMP is a pivotal cytoplasmic mediator of an intracellular signalling cascades that regulate fertilisation in mammalian sperm (Yanagimachi, 1994). The involvement of cAMP in sperm capacitation *in vitro* has been widely studied by several groups (Fraser and Monks, 1990; Harayama and Miyake, 2006; Harrison and Miller, 2000; Leclerc et al., 1996). Although the precise molecular mechanisms involved in sperm capacitation are not fully understood, there is strong evidence that cAMP plays an important role in this process (Fraser and Monks, 1990). In sperm cAMP acts through the activation of PKA (Brautigan and Pinault, 1991) and increased tyrosine phosphorylation in human (Leclerc et al., 1996), bovine (Galantino-Homer et al., 1997) and murine (Visconti et al., 1995b) sperm. It was observed that cAMP increased during capacitation and stimulates sperm hyperactivation motility and also induces acrosome reaction (Eddy and O'Brien 1994; Leclerc et al., 1996; Lefievre et al., 2000; Visconti et al., 1995b).

The intracellular levels of cAMP are regulated by the synthesis from adenosine triphosphate (ATP) through the action of the specific form of SACY and degradation of cAMP by cyclic nucleotide phosphodiesterases (PDEs) to a 5-AMP (Bentley and Beavo, 1992). PDE hydrolyzes the 3, 5-phosphodiester bond of cAMP in order to maintain low levels of cAMP and regulate the effects of this important messenger. This

suggested that PDE plays an important role in the control of cyclic nucleotide levels in spermatozoa. The effect of PDE enzymes was reversed by the addition of some inhibitors such as 3-isobutyl-1-methylxanthine (IBMX), caffeine, and pentoxifylline, and of specific inhibitors of types 1 and 4 PDE. These compounds were shown to increase capacitation and protein tyrosine phosphorylation *in vitro* (Galantino-Homer et al., 1997; Leclerc et al., 1996; Visconti et al., 1995b), motility (Fisch et al., 1998), and acrosome reaction (Fisch et al., 1998; Tesarik et al., 1992) in spermatozoa.

In addition, several studies have investigated the effect of cAMP by adding various types of pharmacological compounds of cell permeant cAMP analogs to the in vitro capacitation media. These analogs such as dibutyl-cAMP (dbcAMP) which is usually added in combination with the PDE inhibitor IBMX (because the dbcAMP is a substrate for the PDE enzymes) and another cAMP analog is sp-cAMP which is resistant to the PDE effect. The addition of cAMP analogs to the capacitation media was found to increase sperm capacitation and accelerate the inhibition caused by the removal of BSA, bicarbonate and Ca^{2+} (Leclerc et al., 1996; Visconti et al., 1995b).

Although few studies have investigated the changes of S/T protein phosphorylation during sperm capacitation, it was widely accepted that reactive oxygen species (ROS) are involved in different capacitation events such as the increase in cAMP levels and activation of cAMP/PKA pathway (Aitken et al., 1998; Leclerc et al., 1997). Interestingly, the regulation of S/T protein phosphorylation during capacitation was found to be modulated by a low level of reactive oxygen species (ROS) including $O2^{-}$, NO and H_2O_2 (O'Flaherty et al., 2004). The participation of these ROS in this finding was upstream of the PKA activation.

1.8.7 Protein kinase A (PKA) in capacitation

Several protein kinases have been involved in sperm capacitation but protein kinase A (PKA) was the most characterized kinase in different species. It is a tetrameric enzyme containing two regulatory (R) and two catalytic (C) subunits. It was demonstrated that PKA activity progressively increased in epididymal mouse sperm during capacitation and also R and C subunits of PKA were found in head, midpiece and principal piece of mouse sperm (Visconti et al., 1995b).

The signal pathway of PKA during capacitation is downstream of cAMP and could be activated by the cAMP activator or inhibited by the cAMP inhibitors. Therefore, inhibition of cAMP will affect PKA pathway and prevent capacitation signalling pathways downstream of cAMP. Moreover, PKA can be blocked by specific inhibitors such as H89 and Rp-adenosine-3-5-monophosphate (Rp-cAMP). Additions of these inhibitors to the capacitation media are able to inhibit the capacitation and also the related tyrosine phosphorylation (Galantino-Homer et al., 1997; Leclerc et al., 1996).

1.9 Protein phosphorylation in sperm cells

Protein phosphorylation is a post-translational modification of proteins that allows the cell to control several cellular processes such as transduction of extracellular signals and intracellular transport and cell cycle progression (Johnson and Barford, 1993). In sperm cells, protein phosphorylation plays an important role in function and regulates processes such as capacitation, hyperactivation motility and the acrosome reaction. Protein phosphorylation occurs predominantly on serine (S) / threonine (T) or tyrosine (Y) residues which is regulated by several kinases and phosphatases (Manning

et al., 2002). The protein kinases transfer a phosphate group from the ATP to a given protein, typically at serine (S), threonine (T), or tyrosine (Y) residues whereas the protein phosphatases catalyze the removal of phosphate groups from specific residues returning proteins to non phosphorylated conformation (Sun and Tonks, 1994). Protein kinases and protein phosphatases are classified into; S/T-specific, Y-specific and dual-specificity kinases and phosphatases (Sun and Tonks, 1994).

Although protein phosphorylation is regarded as a hallmark and appears to be essential for sperm capacitation, the phosphorylation specific cell signalling is complex and not well understood. However, numerous studies have investigated the changes of protein phosphorylation during capacitation. Most of the changes in protein phosphorylation have been studied during sperm capacitation was in tyrosine residues. In contrast, S/T protein phosphorylation in sperm capacitation and other events have been less studied than that of tyrosine residues and need more investigation.

1.9.1 Protein tyrosine phosphorylation during capacitation.

Protein phosphorylation is an important posttranslational event involving multiple levels of cellular regulation. One of the key intracellular events during capacitation is protein tyrosine phosphorylation. The first immunological and biochemical report of tyrosine phosphorylation proteins in mammalian sperm was reported by (Berruti and Martegani, 1989). They investigated protein tyrosine phosphorylation by comparing epididymal and mature (ejaculated) boar sperm lysates. Tyrosine phosphorylated proteins were found to be increased in the mature sperm compared with the epididymal sperm and this finding suggested an effect on these proteins during transportation from the epididymus to the ejaculates. This finding was

the start point for the establishment of the several investigations on protein tyrosine phosphorylation pathway in mammalian sperm and the possible role of this kind of protein phosphorylation during fertilisation.

Protein tyrosine phosphorylation of mammalian sperm can be affected by bicarbonate, Ca^{2+}, and albumin levels in the *in vitro* media (Visconti et al., 1995a; Visconti et al., 1995b). This type of protein phosphorylation is associated with certain aspects of sperm physiology. Bicarbonate activates SACY that is abundant in sperm and acts as a bicarbonate sensor to generate cAMP (Chen et al., 2000; Okamura et al., 1985). The subsequent activation of protein kinase A (PKA) (Flesch and Gadella, 2000) induces, via an as yet unknown signalling mechanism, tyrosine phosphorylation of several proteins in boar (Flesch et al., 1999; Harayama, 2003; Kalab et al., 1998), mouse, bull and humans [for example see (Galantino-Homer et al., 1997; Leclerc et al., 1996; Visconti et al., 1995a), respectively].

During capacitation, sperm hyperactivation depend on cAMP produced by the activation of SACY which is in turn stimulated by bicarbonate and Ca^{2+} (Carlson et al., 2007; Chen et al., 2000). The cAMP generated by SACY stimulates PKA that phosphorylates serine or threonine residues on proteins which activate tyrosine phosphorylation via unknown mechanism (Visconti and Kopf, 1998; Visconti et al., 2002). It was postulated that increased tyrosine phosphorylation in the sperm flagellum induced hyperactivated motility in hamster (Si and Okuno, 1999) but not in bull (Marquez and Suarez, 2004). SACY knock out mice (Esposito et al., 2004) and PKA catalytic subunit knock out mice (Nolan et al., 2004) are infertile due to failure hyperactivation and lack in bicarbonate induced capacitation responses. The SACY

knock out infertility was bypassed by the addition of cAMP analogs to the in vitro capacitation media.

Although sperm tyrosine phospho proteins have been identified in many species, their roles and regulation during capacitation are still not fully understood (Luconi et al., 1996; Visconti and Kopf, 1998). Thus, few studies investigated the role of tyrosine phosphorylation during capacitation using tyrosine kinase inhibitors and yielded controversial results (Carrera et al., 1996; Pukazhenthi et al., 1998; Thundathil et al., 2002). In addition, the inhibitors of PKA activity are able to inhibit tyrosine phosphorylation as well as capacitation (Leclerc et al., 1996; Visconti et al., 1995b). It has been hypothesized that PKA phosphorylates some enzymes/proteins as an intermediate step and that these phosphorylated PKA substrates are involved in capacitation-related protein tyrosine phosphorylation and its regulation (Aitken et al., 1998; Visconti et al., 2002).

During capacitation, most phosphotyrosine proteins become largely localised to the flagellum suggesting that this regulates sperm motility and may be necessary for the induction of hyperactivated motility which has been demonstrated in the mammalian species studied to date i.e. boar (Holt and Harrison, 2002), mouse (Urner et al., 2001), human (Carrera et al., 1996; Leclerc et al., 1996; Sakkas et al., 2003), dog (Petrunkina et al., 2003) and rat (Lewis and Aitken, 2001). This localisation of the tyrosine phosphorylated proteins in sperm flagellum is important to understand the possible corresponding function of these different proteins. Interestingly, the most tyrosine phosphorylated proteins were localised using immunofluorescence and were located to the principle piece which increased by the incubation time and precedes to

the midpiece when sperm bind the zona pellucida in human (Carrera et al., 1996; Nassar et al., 1999; Sakkas et al., 2003) boar (Harayama et al., 2004a), hamster (Si and Okuno, 1999). This increase of tyrosine phosphorylation proteins to the principal piece has been correlated with hyperactivated motility in human (Nassar et al., 1999) and hamster (Si and Okuno, 1999) in order to penetrate the zona pellucida and fertilise the oocyte.

The identity and localisation of the protein substrates for tyrosine phosphorylation are important to determine the various signal transduction pathways in sperm. Several studies have reported protein tyrosine phosphorylation in different sperm species during capacitation, human (Leclerc et al., 1996), bovine (Galantino-Homer et al., 1997) and hamster (Visconti et al., 1999a). However, to date, only a few sperm-phosphorylated proteins have been identified and the knowledge of sperm tyrosine kinases remains limited and many of these proteins have not been identified yet. Few studies have identified tyrosine phosphorylated proteins in the sperm flagellum. Remarkably, a study by Carerra et al, identified tyrosine phosphorylated proteins containing A kinase anchor protein (AKAP4) which was correlated to the hyperactivated motility in sperm during capacitation (Carrera et al., 1996).

In human sperm, two AKAP4 proteins were phosphorylated in tyrosine residues with molecular weights 97 and 82 kDa and were localised to the fibrous sheath of the principal piece (Carrera et al., 1996). The tyrosine phosphorylation of this AKAP4 protein was blocked by a general tyrosine kinase inhibitor. Moreover, tyrosine phosphorylated proteins in fibrous sheath protein of 95 kDa (FS95) was the second fibrous sheath protein that has been cloned and localised to the cytoplasm of the

principal piece of the human sperm tail during capacitation which is later defined as A kinase anchor protein 3 (AKAP3) sperm flagellumr protein (Ficarro et al., 2003; Mandal et al., 1999; Vijayaraghavan et al., 1999). Naaby-Hansen et al, has identified an acidic (pI 4.0) 86-kDa isoform of a novel, polymorphic, testis-specific protein, designated calcium-binding tyrosine phosphorylation-regulated protein (CABYR), that was tyrosine phosphorylated during in vitro capacitation (Naaby-Hansen et al., 2002). This protein is localised to the principal piece of the human sperm and acquires the capacity to bind to calcium during capacitation which is involved in the regulation of sperm hyperactivated motility (Ho and Suarez, 2001a). A fourth tyrosine phosphorylated protein identified during capacitation of mouse, rat and human sperm was a heat shock protein 90 (HSP-90) (Ecroyd et al., 2003).

The activation of tyrosine phosphorylation in the sperm tail proteins is necessary for sperm binding to the zona pellucida but sperm displaying tyrosine phosphorylation in the acrosomal proteins was not increased during capacitation in mouse (Leyton et al., 1992; Urner et al., 2001). Capacitation associated redistribution of phosphotyrosine residues to the acrosome has been reported in boars (Petrunkina et al., 2001; Tardif et al., 2001), bulls (Cormier and Bailey, 2003) and buffalo (Roy and Atreja, 2008). Furthermore, a specific phosphotyrosine protein localised over the acrosomal region has been postulated to be involved in the zona pellucida interaction and/or fusion events (Dube et al., 2005; Ficarro et al., 2003). Additionally, It was reported that tyrosine phosphorylation plays an important role in human acrosome reaction (AR) induced by recombinant human zona pellucida 3 (ZP3) (Brewis et al., 1998; Burks et al., 1995). Therefore, it is possible that assessment of tyrosine phosphorylation in sperm using indirect immunofluorescence *in vitro* may provide an

assay highly predictive of the ability of sperm to bind to the ZP and undergo the ZP-induced AR processes critical for human fertilization. The distribution of phosphotyrosine residues and their modifications associated to sperm capacitation might indicate a role of these proteins during capacitation and in acrosome reaction regulation (Asquith et al., 2004).

1.9.2 Serine and threonine (S/T) phosphorylation

Many studies have investigated tyrosine phosphorylation in mammalian sperm, even though it is far less frequent in vertebrate somatic cells serine/threonine protein phosphorylation (1: 1800: 2000 for Tyr: Ser: Thr phosphorylation events) (Hunter, 1998). In contrast, there have been very few studies on S/T protein phosphorylation in sperm and our understanding is therefore very limited. One major reason for this inequality is the general availability of antibodies that recognise phosphorylated tyrosine residues whereas good quality antibodies which specifically recognise phospho (S/T) proteins/peptides have only become available in the recent years. These new reagents are typically antibodies generated against the consensus sequences of individual kinases, for example, the PKB/Akt pathway and represent useful reagents to survey the dynamics of S/T phosphorylation (Alessi, et al., 1996).

Very little is known about S/T protein phosphorylation but several studies have shown a time dependent increase in serine and/or threonine phosphorylation during sperm capacitation in species such as humans (O'Flaherty et al., 2004), hamsters (Jha and Shivaji, 2002b), boars (Harayama, 2003), ram (Grasa et al., 2009) and mice (Jha et al., 2006). It is noteworthy that this increase in S/T protein phosphorylation appears to occur early during capacitation, as reported in boar for PKA-catalysed phosphorylation

(Harayama, 2003; Harayama and Nakamura, 2008; Harrison, 2004) or in mouse proline-directed S/T phosphorylation (Jha et al., 2006) which would indicate that the activation of S/T kinases is upstream of tyrosine phosphorylation. More recently, specific targets of PKA phosphorylation have been established in human (Moseley et al., 2005; O'Flaherty et al., 2004) and boar sperm (Harrison, 2004). Likewise, a cAMP-independent S/T phosphorylation has been described. An ecto-cAMP independent protein kinase has been located on the outer surface of mature goat spermatozoa (Maiti et al., 2004) and a proline-directed S/T phosphorylation associated to mouse sperm capacitation has been reported (Jha et al., 2006). The effect of cAMP on S/T protein phosphorylation was studied in boar and showed that the addition of the cAMP analog to the boar sperm increased S/T protein phosphorylation via activation of PKA in the principal piece which may be correlated to the hyperactivation motility caused by capacitating agent induced cAMP (Harayama and Nakamura, 2008). This S/T protein phosphorylation was earlier than that of tyrosine.

In contrast to tyrosine phosphorylated proteins, studies carried out to date on serine and threonine protein phosphorylation are relatively scarce. However, a preferential localisation of S/T phosphorylated proteins over the tail regions has been reported in capacitated sperm of humans (O'Flaherty et al., 2004), hamsters (Jha and Shivaji, 2002b) and mice (Jha et al., 2006). The localisation in the human sperm flagellum incubated under capacitating conditions reported by O'Flaherty et al, using antibody against Arginine-X-X-(phospho-S/T) PKA substrate motif was not restricted to the principal piece (O'Flaherty et al., 2004). Controversially, the threonine protein phosphorylation detected by an antibody raised against thr-glu-tyr was localised to the principal piece of the human sperm flagellum during capacitation (Thundathil et al.,

2003). This discrepancy may be related to the different antibody motifs used to detect this kind of protein phosphorylation and also to the dual phosphorylation in the latter motif with both tyrosine and threonine amino acids.

1.9.3 Serine/threonine (S/T) phosphatases

Signalling events leading to mammalian sperm capacitation rely on activation/deactivation of protein phosphorylation. These events were controlled by two parallel pathways including an activation of PKA and/or inactivation of S/T phosphatases. The role of the S/T phosphatases in sperm during capacitation was especially on the hyperactivity of sperm flagellum motion in several species (Yanagimachi, 1994). Eukaryotic protein phosphatases are classified into two distinct gene families: S/T protein phosphatases and phosphotyrosine phosphatases (PTP) (Barford et al., 1998). Protein phosphatases specific for phospho (S) and phospho (T) residues are involved in virtually all aspects of cellular regulation and catalyse the release of phosphates from (S) and (T) residues of the protein substrates (Cohen et al., 1990).

Based on biochemical parameters, S/T protein phosphatases were initially divided into two classes: type-I protein phosphatases (PP1) are inhibited by two heat-stable proteins, termed inhibitor-1 (1-1) and inhibitor-2 (1-2), whereas type-2 phosphatases are insensitive to the heat-stable inhibitors (Cohen, 1989). Type-2 phosphatases can be further subdivided into spontaneously active (PP2A), Ca^{2+}-dependent (PP2B) and Mg^{2+}- dependent (PP2C) classes. The PP1 is highly conserved in all eukaryotes and it controls a variety of processes, such as cell division, transcription, translation, muscle contraction, glycogen and lipid metabolism, neuronal

signalling and embryonic development (Ceulemans and Bollen, 2004; Wera and Hemmings, 1995). Investigations have been ruled out using different pharmacological inhibitors such a Calyculin A, okadaic acid and calcinurine to assess the effect of these inhibitors on different sperm events during sperm capacitation (Adachi et al., 2008; Goto and Harayama, 2009; Han et al., 2007; Huang et al., 2005; Jha and Shivaji, 2002a; Tash et al., 1988). These phosphatase inhibitors were able to block protein phosphatases and increased the phosphorylation of S/T proteins.

1.10 Other signalling studies of protein phosphorylation in sperm capacitation

Protein kinases play a pivotal role in intracellular signal transduction systems. In addition to the well-characterized protein kinase A (PKA) in sperm protein phosphorylation (Visconti et al., 2002), few other S/T kinases have been involved in the regulation of the mammalian sperm capacitation, hyperactivated motility, interaction with the zona pellucida and the acrosome reaction (de Lamirande and O'Flaherty, 2008; Salicioni et al., 2007; Tulsiani et al., 2007). Some of these protein kinases include protein kinase C (PKC) (Ashizawa et al., 2006; Harayama and Miyake, 2006), glycogen synthase kinase (GSK) (Aparicio et al., 2007a; Vijayaraghavan et al., 1996), PKB/Akt (Aparicio et al., 2007b; Nauc et al., 2004) and the extracellular signal-regulated kinase (ERKs) (de Lamirande and Gagnon, 2002; Liguori et al., 2005; Luconi et al., 1998).

1.10.1 Phosphoinositide 3-kinase (PI3K)

PI3K is a heterodimeric protein consisting of a p85 regulatory (adaptor) subunit and a p110 catalytic subunit (Cantrell, 2001). PI3K is implicated in many biological processes, including cell survival and chemotaxis, membrane ruffling and DNA

synthesis, receptor internalization, and vesicular trafficking (Cantrell, 2001; Wymann and Pirola, 1998). In somatic cells, PI3K also phosphorylates a large spectrum of protein substrates (Wymann and Pirola, 1998)

The role of phosphoinositide 3-kinase (PI3K) during sperm capacitation was investigated using a specific inhibitors; LY294002 and wortmannin which affected the level of Ca^{2+} and S/T protein phosphorylation differently (Nauc et al., 2004). Akt or protein kinase B is a S/T protein kinase, downstream of PI3K. PKB/Akt was related to the protein phosphorylation activation/deactivation of substrates involved in cell survival and metabolism and also affects ERK1/2 (Perkinton et al., 2002).

1.10.2 Extracellular signal-regulated kinase (ERK2)

Extracellular signal-regulated kinase (ERK2) is an isoform of the mitogen activated protein kinase family (MAPK) which plays a role in capacitation (de Lamirande and Gagnon, 2002) and acrosome reaction (Luconi et al., 1998). An increase in phosphorylation of the thr-glu-tyr motif of proteins from human sperm incubated in capacitation media suggested that ERKs are activated by the dual specificity kinase MEK which appears to play a necessary role during capacitation (de Lamirande and Gagnon, 2002). ERK2 was activated by progesterone in human sperm and redistributed from the post-acrosomal region to the equatorial segment (Luconi et al., 1998). This localisation of phosphorylated proteins may indicate that this pathway is required to control protein phosphorylation on the sperm head.

1.10.3 Protein kinase C (PKC)

Protein kinase C (PKC) is present in sperm and may be activated during sperm capacitation (Harayama and Miyake, 2006) and acrosome reaction (Ashizawa et al., 2006). Immunolocalisation studies have shown that PKC localised mainly to the equatorial segment of human sperm (Rotem et al., 1990), to the upper region of the acrosome in ram sperm (Breitbart et al., 1992) and to the post acrosomal and connecting regions in boar sperm (Harayama and Miyake, 2006). This localisation of PKC in the sperm head may indicate a possible role of this kinase in acrosome reaction. Moreover, PKC was found to be regulated by the increase of cAMP leading to a hyperactivation of boar sperm which was also inhibited by addition of PKC specific inhibitor and subsequently blocked hyperactivation (Harayama and Miyake, 2006).

1.10.4 Glycogen synthase kinase-3 (GSK3)

Glycogen synthase kinase-3 (GSK3) is one of several S/T kinases with two isoforms α and β expressed in mammalian tissues (Woodgett, 1990). This kinase was found to phosphorylate and inactivate glycogen synthase enzyme, the final enzyme in glycogen biosynthesis (Frame et al., 2001). In contrast to other kinases, GSK3 activity is reduced by the phosphorylation of S/T (Frame et al., 2001), and it is activated by tyrosine phosphorylation (Tardif et al., 2001). GSK3 has been described in sperm including bovine (Vijayaraghavan et al., 1996), which showed that the immotile caput sperm contains sixfold higher GSK3 activity than the caudal sperm. A year later, an increase of tyrosine phosphorylation of 55 kDa was reported in bovine epididymal sperm (Vijayaraghavan et al., 1997a). This 55 kDa protein was found to be correlated to the GSK3 and may be related to the initiation of motility in the epididymus

(Vijayaraghavan et al., 2000). A correlation between the increase of S/T phosphorylation within GSK3 and decreased GSK3 activity has been postulated in bovine epididymal sperm during their passage through the epididymus (Somanath et al., 2004). More recently, both GSK3 isoforms have been described in boar sperm but only α isoform was associated with sperm motility (Aparicio et al., 2007a). The mechanism of motility regulation by GSK3 in sperm needs to be elucidated in future investigations.

1.11 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) describe a group of metabolites that are derived from the reduction of oxygen including free radicals such as superoxide anion (O_2^-) and hydroxyl radical (OH); as well as oxidants like (H_2O_2) and nitric oxide (NO). These free radicals and related ROS are able to react with several kinds of bimolecules in the cells including proteins, lipids and nucleic acids (Aitken and Bennetts, 2006). Sperm are active cells that are capable of generating ROS such as O2 and H_2O_2. This activity is of fundamental biological importance in regulating the signal transduction pathways which control sperm capacitation. In this context, as the sperm plasma membrane is very rich in unsaturated fatty acids, it becomes more susceptible to attack by the ROS (Jones et al., 1979).

It has become clear that ROS generation by mammalian sperm has an important role to play on both capacitation and acrosome reaction. ROS have dual effects on sperm functions depending on its concentrations. At low concentration, it was found to induce sperm capacitation (de Lamirande and Gagnon, 1993; Ford, 2004; Leclerc et al., 1997), whereas excessive concentration of ROS has been associated with the

etiology of defective sperm function and DNA damage (Bennetts and Aitken, 2005; Lopes et al., 1998), inhibit sperm and oocyte fusion (Aitken et al., 1989) and reduce sperm motility (de Lamirande and Gagnon, 1992). The stimulatory effect of ROS on sperm capacitation is believed to be mediated by the induction of tyrosine phosphorylation (Aitken et al., 1995; Aitken et al., 1996). ROS effects on sperm capacitation were associated with other changes caused by other capacitation factors such as bicarbonate and Ca^{2+} which increase intracellular superoxide (O_2^-). The latter, was found to activate adenylyl cyclase in rat sperm (Lewis and Aitken, 2001) and increases cAMP level which as a result induces PKA and tyrosine phosphorylation by inhibiting tyrosine phosphatase. Moreover, It was reported that hydrogen peroxide (H_2O_2) suppressed tyrosine phosphatases activity in human sperm and also induce cAMP generation by adenylyl cyclase which stimulated tyrosine phosphorylation (Aitken et al., 1998).

Nitric oxide (NO) is another oxygen free radical which is generated by defective human sperm. NO is synthesized by the NO-synthase (NOS) enzymes (Palmer et al., 1988). This reaction requires some cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide, calcium and calmodulin (Moncada and Higgs, 1995). Molecular oxygen is used in this reaction to form NO and an intermediate L-citrulline. NO-synthase (NOS) enzyme has been reported in sperm for the first time in two separated studies in 1996, mouse and human (Herrero et al., 1996; Lewis et al., 1996). In mouse sperm, NO was found to be increased within first 120 min of incubation in capacitating media and this may suggest a physiological significance during capacitation (Herrero et al., 1997). NO was suggested to affect both sperm viability and motility, that low concentration of NO

stimulates hyperactivation motility of mouse sperm (Herrero et al., 1994). In contrast, high concentration of NO was found to inhibit sperm motility which may be caused by the sperm respiration inhibition (Weinberg et al., 1995). Consistent with this, high NO concentrations have been found in semen of infertile men with decreased motility which was enhanced by the addition of NOS inhibitors (Perera et al., 1996). NO was also involved with sperm capacitation in a manner similar to that observed with cAMP and consequently enhancing tyrosine phosphorylation (Herrero et al., 1999). Additionally, NO also appears to participate in the AR (Kuo et al., 2000) and zona pellucida binding (Sengoku et al., 1998).

The main aim of this thesis was to investigate and characterise the changes in (S/T) protein phosphorylation during boar sperm capacitation. This was performed by immunublotting and indirect immunfloresence using different phospho (S/T) kinase substrate antibodies. This was the first time that this has been studied in boar sperm. In addition, the model system was also validated by studying sperm motility, viability and tyrosine phosporylation during capacitation.

1.11 Major aims

The major aims of the thesis were to:

- Establish a valid system for assessing boar sperm incubated under capacitating C conditions
- Investigate the changes in S/T protein phosphorylation in boar sperm incubated under C conditions
- Further investigate S/T protein phosphorylation using pharmacological agents in sperm incubated under C conditions
- Perform subcellular fractionation of sperm to localise the changes in S/T protein phosphorylation
- Identify proteins that show changes in S/T phosphorylation in sperm incubated under C conditions

CHAPTER 2

Materials and methods

2.1 Sperm preparation

All chemicals were obtained from (Fischer, Epsom, Surrey, UK) unless otherwise specified. Boar semen ejaculates were collected from Landrace boars and supplied in extender buffer (JSR Healthbred, Southburn, Driffield, York, UK). Sperm were isolated by centrifugation (750 *g* for 20 min with brake off) using (Megafuge 1.0R, Heraeus instruments, Germany) through a two-step discontinuous gradient of 35% and 70% isotonic Percoll (Sigma-Aldrich Company Ltd., Poole, UK) (15 ml of each gradient overlaid with 15 ml semen in extender buffer). Isotonic Percoll was prepared by first mixing Percoll with 10X M medium (1.37 M NaCl, 25 mM KCl, 200 mM HEPES, 100 mM glucose, pH 7.55) (12:1 ratio) and the osmolality checked to confirm it was 295-300 mOsm / kg and pH 7.40± 0.05. This was then diluted to the appropriate concentration with 1X M medium. After centrifugation the Percoll layers were removed and the resultant loose pellet was resuspended in Tris-buffered sucrose solution (TBSS; 5 mM Tris, 0.25 M sucrose, pH 7.4; 10 ml). Sperm were counted using a haemocytometer with a final concentration of 1 x 10^7 / 1 ml and then subjected to further centrifugation (500 *g* for 5 min). The pelleted cells were then resuspended in the appropriate buffer for incubation.

2.2 Sperm incubation

Cells prepared as described above were incubated under either non-capacitating (N) or capacitating C conditions at a concentration of 10^7 / ml in 10 ml media. For (C) conditions Tyrode's medium was used (100 mM NaCl, 21.7 mM lactate, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 15 mM $NaHCO_3$, 100 μg / ml kanamycin AND containing 0.3 % (w/v) bovine serum albumin (delipidated Fraction V, Sigma–Aldrich) with the

cells in a tube with the screw cap loose at 38.5 °C in a humidified incubator with 5% CO_2 in air (Gadella and Harrison, 2000; Parrish et al., 1988). N conditions were the same except that the Tyrode's medium was prepared without $NaHCO_3$ (but with additional 16 mM NaCl to maintain the same osmolality) and samples were incubated in air tight sealed tubes to prevent the formation of bicarbonate. Osmolality was measured for both N and C buffers between 295-298 mOsm (using Digital Micro-Osmometer (Type 5B), Hermann Roebling Messtechnik, Berlin) at Heamatology Department, Cardiff University, Prof Alan Burnett). Typically samples were recovered for immunoblotting, immunofluorescence experiments or other experiments depend on the relevant time for the individual experiment (15 min, 30 min, 1 h or 3 h incubation). Sperm pellets were stored at -20 °C for subsequent use.

2.3 One dimensional electrophoresis (1DE)

Following incubation in either N or C medium, sperm aliquots (1 x 10^7 cells) were collected. These aliquots were centrifuged for 10 min at 5000 *g*, washed in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and again centrifuged for 10 min at 5000 *g*.

One dimensional electrophoresis (1DE) was performed using the Invitrogen mini gel system. The isolated sperm pellet was resuspended in 100 µl sample buffer (NuPAGE LDS, Invitrogen) and heated at 70 °C for 10 min. The sample was recentrifuged at 10,000 *g* for 5 min to remove non-solubilised material. Sperm proteins in the supernatant equivalent to 1.5-2 x 10^6 cells were separated by 1DE at 200 V for 40-45 min on 4-12 % Bis-Tris NuPAGE Novex gels (1.5 mm thickness (Invitrogen). Each electrophoresis run included a set of eight pre-stained molecular weight markers

(SeeBlue, Invitrogen). The position of the markers on the blotted membrane was traced onto the film.

2.4 Immunoblotting

Proteins were transferred onto polyvinylidene fluoride membranes (PVDF, GE Healthcare) which were washed with methanol and immersed in 1X transfer buffer (50 ml NuPAGE transfer buffer 20X (Invitrogen), 100 ml methanol and 850 ml water) at 30 V and 170 A for 1.5 h. Membranes were washed briefly with PBS-Tween 20 and blocked with IBT-Tween 20 (0.2 %(w/v) I-Block, 0.1(v/v) % Tween- 20 and 0.4 % (w/v) sodium azide) for 2 h. Blocked membranes were probed with diluted antibodies of interest overnight (all at 1:100 dilution unless otherwise specified) (Table 2.1). Membranes were washed 3 x 10 min with PBS-Tween 20 and incubated with secondary antibodies for 1 h and washed 3 x 10 min. Previously described alkaline phosphatase chemiluminescent detection protocol (Rowe and Jones, 2001) was used for protein visualisation. Briefly, membranes were washed with alkaline phosphatase buffer (Tropix, Applied Biosystems Warrington, UK) for 2 min and incubated with substrate (tropix, CDP-star, Ready to use; Applied Biosystems; Warington,UK) for 5 min and developed with Kodak MXB blue films (Genetic Research Instrumentation (GRI), Ltd, Braintree, Essex, UK).

For loading consistency, all membranes were stripped with MESNA stripping buffer (62.5mM Tris pH 6.8, 0.82 g sodium 2-mercaptoethanole sulfonate; Sigma, 2 g SDS; Amersham) for 30 min at 50 °C. Stripped membranes were washed 3 x 10 min with PBS and incubated for 1 h with α-tubulin antibody (1:100; Sigma-Aldrich) then

washed 3 x 10 min and probed with anti-mouse secondary antibody for 1 h (Bio Rad Laboratories, Bio-Rad House, Hamel Hempstead, Hertfordshire, UK).

Primary Ab	Source	Peptides or motif recognised	Immunoblotting dilution	Company
anti-phosphotyrosine (clone 4G10)	Mouse (monoclonal)	Tyrosine phosphorylated proteins	1:1000	Upstate (Croxley Green Business Park, Watford, UK)
Phospho (S/T) Akt substrate	Rabbit (monoclonal)	(R/K-X-R/K-X-X-T*/S*)	1:1000	Cell Signalling Technology Cat#9611 (Hitchin, Hertfordshire, UK)
Phospho (S/T) Akt substrate	Mouse (monoclonal)	(R-X-R-X-X-T*/S*)	1:1000	Cell Signalling Technology Cat.# 9614
Phospho (S/T) ATM/ATR substrate	Rabbit (polyclonal)	(Hyd-S*/T*-Q)	1:1000	Cell Signalling Technology Cat#2851
Phospho (S/T) PKA substrate	Rabbit (monoclonal)	(R-R-X-S*/T)	1:1000	Cell Signalling Technology Cat #9624
Phospho (S) PKC substrate	Rabbit (polyclonal)	(R/K-X-S*-Hdy-R/K)	1:1000	Cell Signalling Technology Cat #2261
Anti-α-tubulin	Mouse (monoclonal)	Tubulin	1:1000	Sigma Aldrich Company Ltd (Gillingham ,Dorset, UK) Cat#T9026
Acr-2 (ab1900)	Mouse	Porcine acrosin	1:1000	Abcam (Science Park, Cambridge, UK)
AKAP3 (ab19046)	Goat	Synthetic peptide: TPLQLLDWLMVNL to C-terminal amino acids 841-853 of human AKAP3	1:1000	Abcam (Science Park, Cambridge, UK)
AKAP4 (AKAP82 F-16) sc-66308	Goat	Raised against a peptide mapping near the C-terminal amino of human AKAP82	1:500	Santa Cruz Biotechnology, INC (UK)
HSP70/HSP72mAb (C92F3A-5)	Mouse	Native human HSP70 protein	1:1000	Stressgen (Enzo Life Sciences LTD ,Matford Court , Exeter, UK)
HSPA1L ab77155	Mouse	Recombinant fragment: KGKISESDKNKILDKCNELL SWLE VN QL AE DEFDHKRKE LEQM CNP II TK LYQGGCTGP ACGTGYVP GRP AT GP T IEEV D. corresponding to amino acids 561-641 of Human HSPA1L	1:1000	Abcam (Cambridge, UK)
Calicin ab75208	Rabbit	A synthetic peptide corresponding to a region of human calicin	1:1000	Abcam (Cambridge, UK)
AKAP3 (C20)sc-47788	Goat	Raised against a peptide mapping near the C-terminal amino of human AKAP3	1:500	Santa Cruz Biotechnology, INC
18.6	Mouse (monoclonal)	Acrosomal contents	neat	A gift from Professor Harry Moore (University of Sheffield)

Table 2.1: Antibodies used to detect proteins in boar sperm.

2.5 Indirect immunofluorescence (IIF)

Incubated sperm cells (1 x 10^5) were gently smeared onto a microscopic slide and allowed to air dry. Slides were fixed in 100 % methanol for 5 min and allowed to air dry for 1 h. Slides were then incubated for 1 h with different primary antibodies (all at 1:100 dilutions unless otherwise specified) at 38.5 °C in a wet box. Following incubation the slides were rinsed with PBS and incubated for a further 1 h with suitable secondary antibody. For each primary antibody used a control slide was processed and analysed where the primary antibody incubation was excluded and the cells were just incubated with secondary antibody (all at 1:300 dilutions unless otherwise specified). Slides were washed with PBS and mounted with Slow Fade Light antifade solution (Dako UK Ltd., Ely, UK). Slides were assessed by Leica epifluorescence microscopy at 492 nm wavelength with 40X oil objective lens magnification to determine the localisation of the detected fluorescence.

CHAPTER 3

Validation of the model system for boar sperm capacitation

3.1 Introduction

Why use boar sperm as a model system?

Boar sperm offer clear advantages as a model to study signalling pathways due to several reasons. These include the very high counts of boar sperm cells compared with human sperm (Gadella, 2009). Ejaculated boar sperm can be stored for up to a week in extender buffers and used for *in vitro* capacitation. This is in contrast to rodent sperm which can also only be humanely obtained from the epididymis. As sperm undergo epididymal maturation to achieve fertilising ability this is not an ideal sperm model (Cooper, 2007). Moreover boar sperm cells are poorly motile on release from the male reproductive tract until stimulated by the addition of bicarbonate which is the capacitating agent for boar sperm (Harrison, 1996). Finally, there are ethical limitations for this type of study in human whilst boar sperm can be readily obtained from artificial insemination centres.

Fertilisation is a very complex event comprising a number of steps and is preceded by sperm capacitation which enables the sperm cell to fertilise the egg. Different definitions of capacitation have led to a situation in which some basic questions about capacitation remain unanswered. For example, what is meant when we say that we are using capacitated sperm? How can the stage of sperm capacitation be measured? In a sperm preparation that is termed capacitated, what proportion of the sperm are capacitated? To date, there is no ideal method to measure sperm capacitation. Moreover, the ideal probe to assess sperm capacitation should not interfere with sperm physiology and should be easily available. Unfortunately, such an ideal compound does not exist.

Hyperactivated motility has been reported in sperm during capacitation and is perhaps the most easily observed function (Ho and Suarez, 2001b; Suarez, 2008; Suarez et al., 1992). It is the most widely used sperm assessment method, as it is also a good indicator of membrane intactness. This hyperactivated motility can be assessed using Computer Assisted Semen Analysis (CASA) instruments which monitors the movement of the sperm head. In this method, the development of sperm hyperactivated motility during capacitation is possible (Mortimer and Mortimer, 1990; Mortimer et al., 1997). The aspects of sperm movement analysed by CASA include the velocity of motion of the sperm head, such as curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP), as well as the amplitude of lateral head displacement about the direction of travel (Burkman, 1991; Mortimer and Swan, 1995; Mortimer et al., 1997). This approach has been used to identify hyperactivated sperm in human sperm in particular. Although sperm hyperactivated motility can be recorded during capacitation, alone it is not an accurate parameter to estimate the percentage of capacitated sperm. Therefore, in this study sperm motility was assessed after incubation in non-capacitating (N) and capacitating (C) medium under light microscopy because there is no CASA available in the laboratory.

Sperm viability is a valuable quality parameter which is measured by permeability of the plasma membrane to specific stains (Nagy et al., 2004). In this study, boar sperm viability was assessed after the incubation in N and C medium to confirm that the incubation medium components did not induce boar sperm death and also to ensure that the sperm cells used are intact and able to undergo capacitation.

Although differentiating C from N sperm remains an inexact science, there are two common methods have been used to assess sperm capacitation. These two methods are the chlortetracycline assay (CTC) (Fraser et al., 1995; Mattioli et al., 1996; Wang et al., 1995; Ward and Storey, 1984) and membrane fluidity assay using merocyanine-540 (Harrison, 1996; Harrison et al., 1996; Williamson et al., 1983). CTC is a fluorescent antibiotic that binds to mammalian sperm, with the pattern of fluorescence in the sperm head reflecting the functional state of the cell. This stain was used as a fluorescence probe to visualise the course of capacitation and the acrosome reaction in the mouse (Saling and Storey, 1979), bull (Fraser et al., 1995), ram (Gillan et al., 1997) and boar (Mattioli et al., 1996; Wang et al., 1995). However, the mechanism for the changes in the staining patterns is not clear.

On the other hand, merocyanine 540 is a lipophilic molecule which contains a negative charged sulphonate group. Merocyanine 540 stains cell membranes more intensely if their lipid components are in a higher disoder (Langner and Hui, 1993; Williamson et al., 1983). In this respect, merocyanine 540 has been used to monitor alterations in the lipid architecture of boar sperm plasma membrane during capacitation (Harrison et al., 1996). The advantage of using merocyanine 540 compared with CTC is that it is more suitable for assessing capacitation flow in a cytometric fashion because this technique allow for analysis of larger numbers of unfixed sperm cells (Flesch et al., 1998). Moreover, merocyanine 540 assay which is a marker for increased membrane fluidity is preferable to CTC (fluorescence microscopy assay), because flow cytometric assays are easier, quicker, no fixation and washing steps and more accurate (Rathi et al., 2001). We considered using

merocyanine 540 to assess capacitation in this thesis as it is perhaps the current method of choice as a capacitation assay. However it was not possible to use it due to the time of incubation required which is one hour and we were more interested in earlier time points.

The other parameter commonly used to assess capacitation is the acrosome integrity during capacitation. It is well known that the AR can proceed only after capacitation (Saling and Storey, 1979). The AR can be detected in many ways, for example using fluorescein isothiocyanate (FITC)-conjugated peanut (*Arachis hyogea*) agglutinin (PNA) as a label (Cheng et al., 1996; Flesch et al., 1998). In this thesis, the acrosomal integrity was assessed to confirm the acrosome intact after different treatments using a fluorescence approach by the monoclonal antibody 18.6 (Brewis et al., 1996).

Finally, an increase of 32 kDa tyrosine phosphorylation and localisation to the principal piece of boar sperm during capacitation has previously been reported by different studies (Bailey et al., 2005; Flesch et al., 1999; Harayama, 2003; Harayama and Nakamura, 2008; Harayama et al., 2004b; Kalab et al., 1998; Tardif et al., 2001). This observation was chosen to be used as a control for boar sperm capacitation in this study. Sperm were incubated under N and C conditions and probed with an anti-phosphotyrosine Ab. The result then was compared with results from previous studies

3.2 Aims

- Establish a robust model system that may be used to investigate sperm capacitation
- Compare bicarbonate-dependent tyrosine phosphorylation with that previously reported and then use as capacitation marker
- Assess the protein solubilisation regimes used for one (1DE) and two dimensional electrophoresis (2DE)

3.3 Material and methods

3.3.1 Assessment of sperm motility and viability

Sperm motility was assessed by subjective observation. The motility was observed in a 20 µl aliquot of sperm suspension on a slide under a bright-field microscope. Sperm viability was assessed using a LIVE/DEAD® sperm viability kit (Invitrogen, Ltd., Paisley, UK). SYBR14 dye (5 µl; 100 nM final concentration) was added to 1 x 10^6 cells in 1 ml N or C medium following 1 h or 2 h incubation and further incubated for 10 min at 38.5 °C. Propidium iodide (5 µl; 12 µM final concentration) was then added and the cells were incubated for a further 10 min at 38.5 °C. Sperm were evaluated using a Leica fluorescence microscope and a total of 200 cells were counted for each treatment. The viability data was assessed using unpaired students *T*-test (Prism software).

3.3.2 Acrosome integrity using 18.6 Ab and nuclear staining by DAPI

Acrosomal status was assessed by indirect immunofluorescence using a monoclonal antibody (designated as 18.6) as described previously (Brewis, et al., 1996). Briefly, 18.6 Ab recognises a sperm epitope within the acrosomal content and fixed cells displaying even fluorescence in the acrosomal region are scored as acrosome intact. Nuclear staining was also performed using 4, 6 dimidine-2-phenylindole dihydrochloride (DAPI; Invitrogen) added to the secondary antibodies (0.2 µl/100 ml).

3.3.3 Boar sperm lysis with different buffers

The sperm pellet (5 x 10^6) was resuspended in 1ml 2DE lysis CHAPS buffer (7M urea, 2 M thiourea and 2% (w/v) CHAPS) and lysed for 45 min at room temperature. The sample was recentrifuged at 10,000 *g* for 5 min. The pellet (non-solubilised material) was smeared on a slide and evaluated with a Leica fluorescence microscope.

Sperm pellet (1 x 10^7) was resuspended in 100 µl 1DE LDS sample buffer (NuPAGE LDS, Invitrogen) and heated at 70 °C for 10 min. The sample was recentrifuged at 10,000 *g* for 5 min. The sperm pellet (non-solubilised material) was smeared on a slide and evaluated with a Leica fluorescence microscopy with 20 X objective lens magnification.

3.3.4 Immunoblotting and IIF of tyrosine phosphorylation

Non-capacitating (N) and capacitating (C) sperm proteins equivalent to 1.5-2 x 10^6 cells were separated by 1DE. For immunoblotting the anti-phosphotyrosine monoclonal antibody was used at 1:1000 dilution (clone 4G10) was from Upstate, Millipore UK Ltd., Watford, UK. The secondary antibody used was goat anti mouse IgG (H+L) AP-conjugate (Bio-Rad Laboratories, Hemel Hempstead, UK) at 1:10,000 dilution. For IIF, slides were incubated for 1 h with 1:200 dilution (clone 4G10). The secondary antibody used was goat anti-mouse Alexa Fluor 488–conjugated antibody (1:300 dilution; Invitrogen).

3.5 Results:

3.5.1 Sperm incubated under capacitating conditions (C) resulted in the development of hyperactivated motility

Sperm motility was assessed by bright field microscopy and the majority of sperm incubated in C medium showed rapid progressive motility from 5 min to 1 h incubation (Figure 3.1). The motility increased over time to become hyperactivated after 1 h incubation (Figure 3.2) and reached a plateau after 2 h incubation. From 2 h - 3 h incubation there was an increase of sperm head to head agglutination. In contrast to C sperm, N sperm cells showed no movement through the microscopic field in all experiments but instead demonstrated a vibrational movement ('static vibrance') caused by sperm sticking to the microscope slide due to a lower lateral head displacement than the C cells. This is consistent with the observation by other workers using the pig as a model system (Dr. Bart Gadella, Utrecht University, The Netherlands, personal communication).

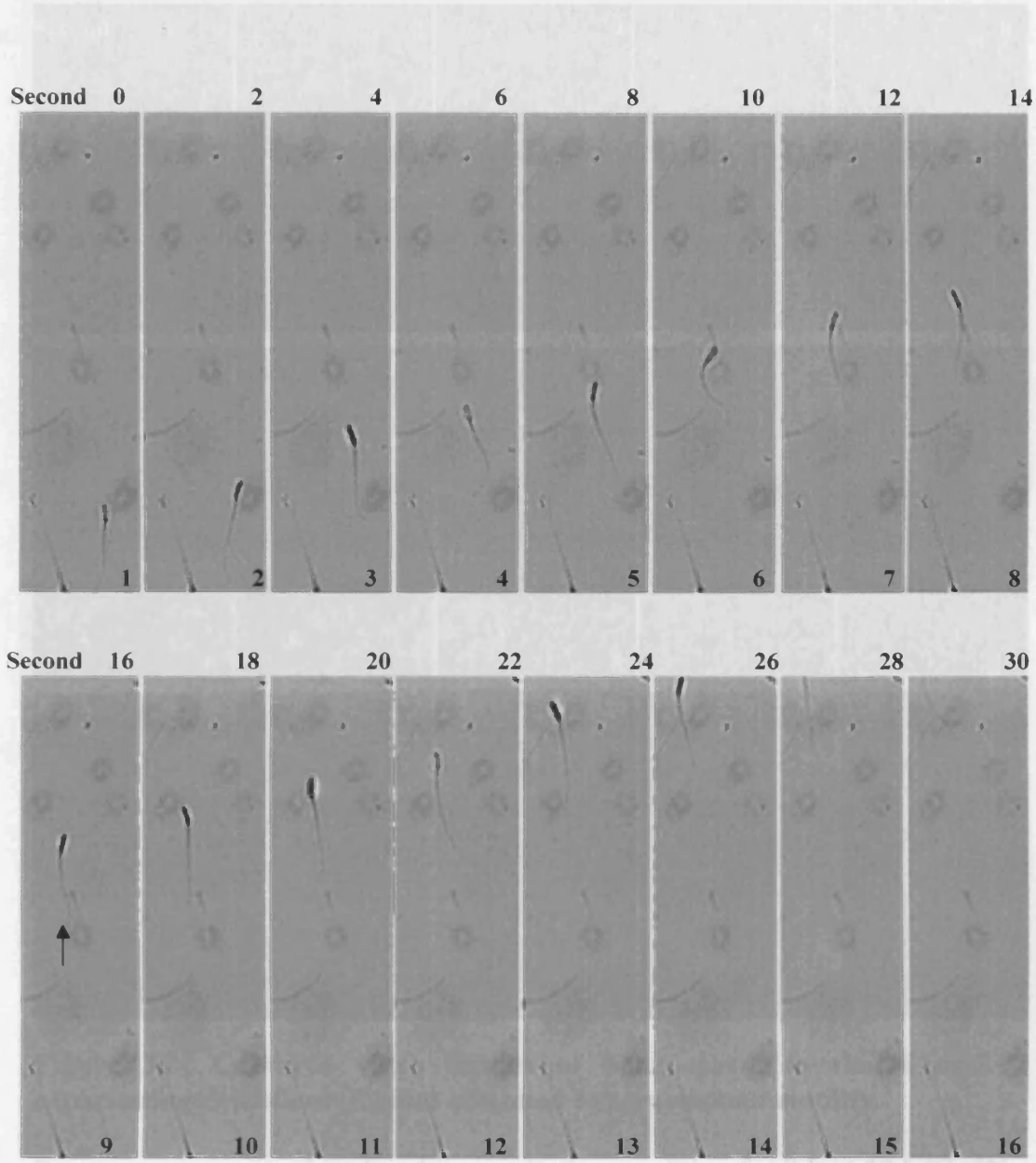

Figure 3.1: Captured video images of boar sperm incubated under capacitating conditions (C) that exhibited progressive motility.

Boar sperm were incubated under capacitating condition for 1 h at 38.5 ºC, 5% CO_2 in air. A 20 µl aliquot of the sperm suspension was observed on a slide under a bright-field microscope. Sperm motility was recorded every 2 seconds (represents successive 2 seconds treatment). Figures from 1-16 show boar sperm progressive motility. The arrow illustrates the sperm cell with progressive motility.

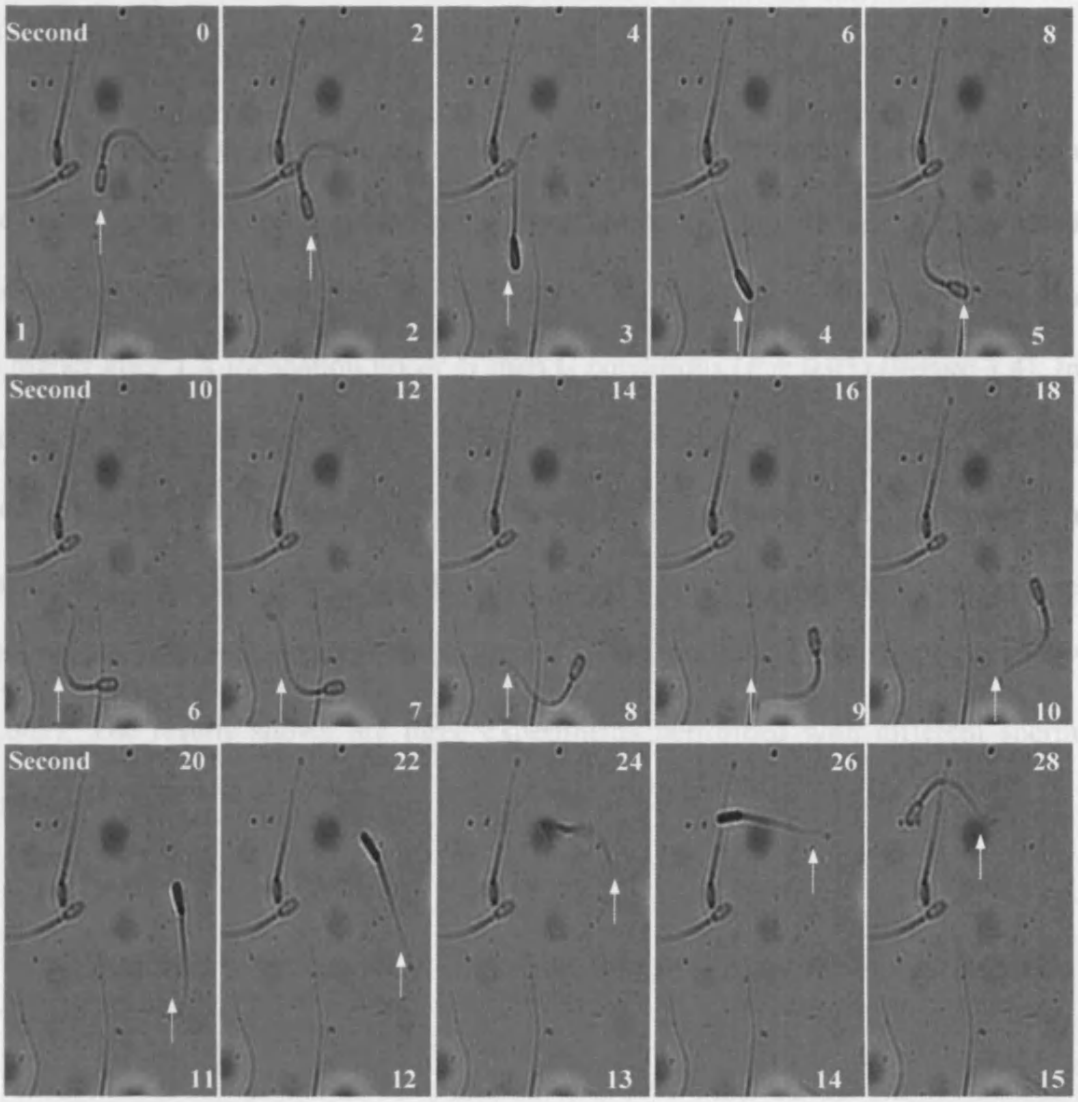

Figure 3.2: Captured video images of boar sperm incubated under capacitating conditions (C) that exhibited hyperactivated motility.

Boar sperm were incubated under capacitating condition for 2 h at 38.5 °C, 5% CO_2 in air. A 20 µl aliquot of the sperm suspension was observed on a slide under a bright-field microscope. Sperm motility was recorded every 2 seconds (represents successive 2 seconds treatment). Figures from 1-15 show boar sperm progressive motility. The arrows illustrate one sperm cell with hyperactivated motility.

3.5.2 Sperm viability was high following incubation in both non-capacitated (N) and capacitated (C) conditions

Viability was assessed using a LIVE/DEAD® sperm viability kit and sperm were classified as live (plasma membrane intact; green Figure 3.3A and 3.3C) or dead (plasma membrane damaged; red Figure 3.3B and 3.3D). The percentage of live sperm was higher after 1 h incubation under N than C conditions ($P = 0.03$) (Figure 3.4). In addition, boar sperm viability was higher under N than C conditions after 3 h incubation ($P = 0.04$) (Figure 3.4). The percentage of live sperm was higher after 1 h incubation under N conditions than that after 3h ($P = 0.02$). Moreover, the percentage of live sperm was higher after 1 h incubation under C conditions than that after 3 h ($P = 0.009$). The results shown are three experiments performed with different sperm samples.

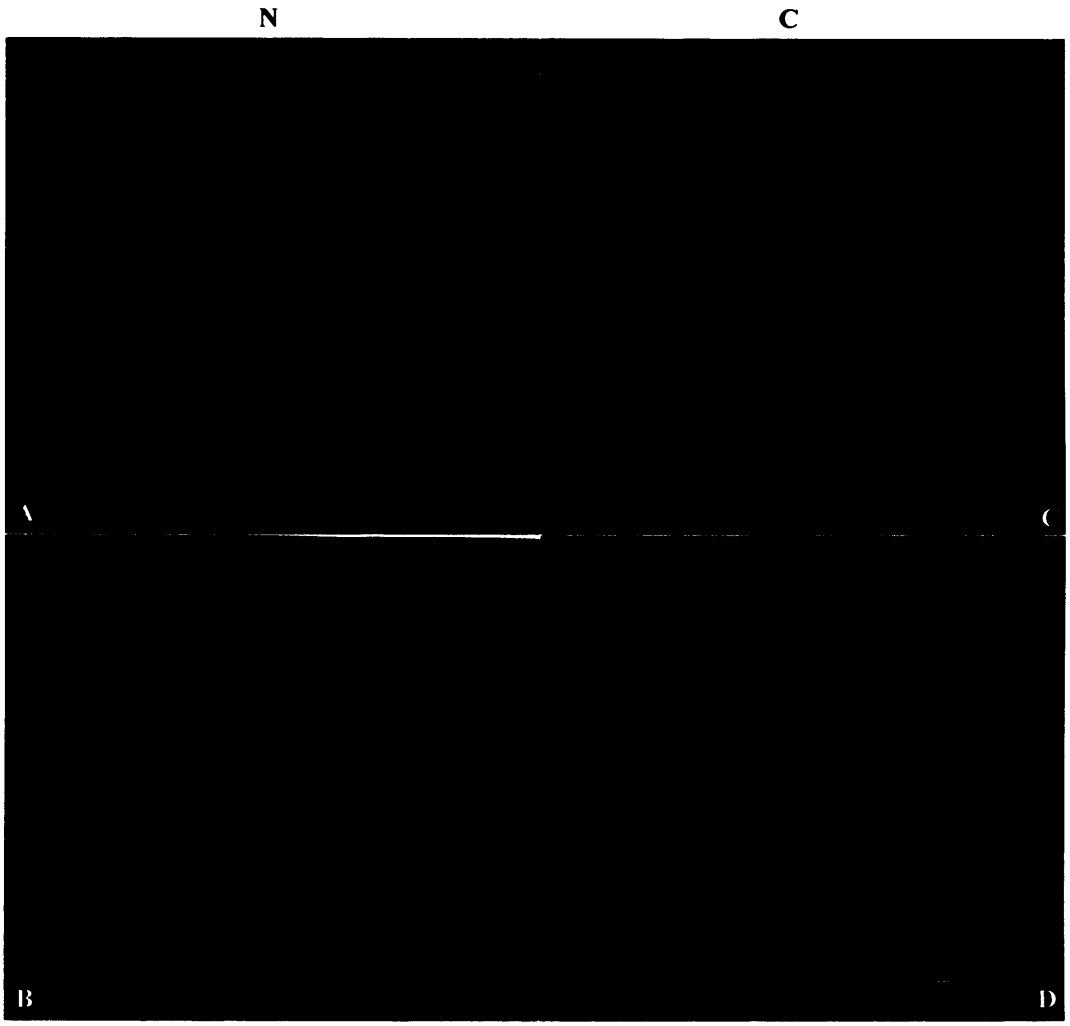

Figure 3.3: Assessment of boar sperm viability after incubation under non-capacitating (N) or capacitating conditions (C) using fluorescence labelling with a LIVE/DEAD sperm viability kit.

(A and **B)** Sperm incubated under N conditions. **(C** and **D)** Sperm were incubated under C conditions. (**A** and **C**) Sperm were labelled with 5 µl SYBR14 at 38.5 °C. (**B** and **D**) sperm were labelled with 5 µl propidium iodide for dead cells.

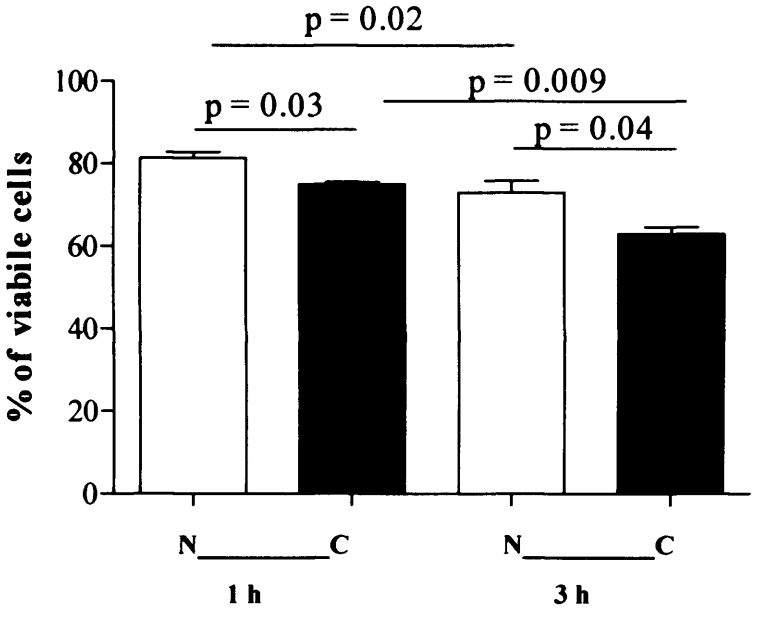

Figure 3.4: Viability in sperm incubated under non-capacitating (N) condition was higher than that of sperm incubated under capacitating (C) conditions after 1 h and 3 h.

Boar sperm were washed and incubated in non-capacitating (N) and capacitating (C) conditions for 1 h **(A)** and 3 h **(B)**. Viability was assessed by SYBR14 dye and Propidium iodide. Sperm were evaluated using a Leica fluorescence microscope and a total of 200 cells were counted for each treatment. The data presented is the mean of three experiments and the error bars are the S.E.M.

3.5.3 No differences in acrosome integrity were observed under N and C conditions

The aim of this section was to assess the acrosome integrity of sperm during centrifugation and different washing steps before it was incubated in N or C buffer. The centrifugation steps included Percoll density gradient centrifugation and washing with TBSS. These steps might affect the quality of sperm and lead to deterioration. This acrosome integrity was assessed using IIF in all experiments by evaluating 100 cells. Sperm were collected immediately before and after centrifugation prior to 1DE and assessed for acrosomal integrity. In all cases at least 90 % of cells were acrosome intact in both N and C non-washed sperm (before centrifugation) (Figure 3.5Aa and Ba) and N and C washed cells (after centrifugation) (Figure 3.5Ab and Bb).

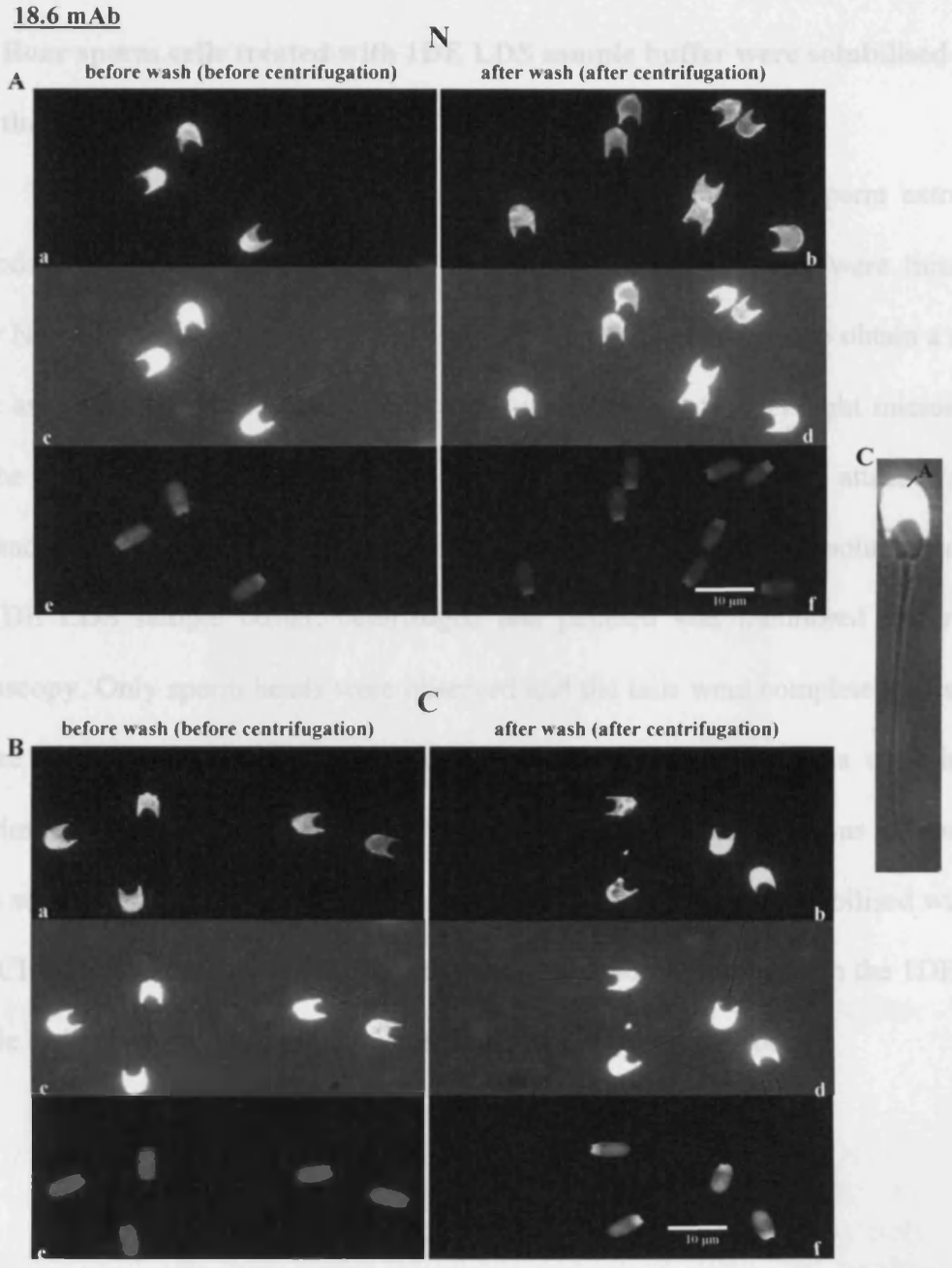

Figure 3.5: The acrosomal integrity of sperm is not affected by centrifugation during the washing steps prior to one-dimensional electrophoresis (1DE).

Sperm were assessed for acrosomal integrity using an indirect immunofluorescence assay with the 18.6 monoclonal antibody. Representative samples are shown of cells before and after washing (before and after centrifugation) following 1 h incubation in (N) and (C) conditions **(A** and **B,** respectively**)**. Triplicate images are shown of the same field of view under fluorescence only for 18.6 **(a and b)** merged fluorescence and light **(c and d)** and fluorescence only for nuclear staining with DAPI **(e and f)**. **(C)** Fixed cells with an acrosome display fluorescence over the acrosome labelled as **A.** The results shown are representative of three experiments performed with different sperm samples.

3.5.4 Boar sperm cells treated with 1DE LDS sample buffer were solubilised more than those with 2DE CHAPS buffer

The aim of this section was to assess the efficiency of the sperm extraction methods with 2DE CHAPS and 1DE LDS sample buffers. Sperm were incubated under N conditions solubilised with 2DE CHAPS buffer, centrifuged to obtain a sperm pellet and smeared onto a microscope slides and monitored under light microscopy. For the cells treated with 2DE CHAPS buffer, sperm heads remained attached to the tails and were not dissolved (Figure 3.6A). On the other hand, sperm solubilised with the 1DE LDS sample buffer, centrifuged and pelleted was monitored under light microscopy. Only sperm heads were observed and the tails were completely dissolved (Figure 3.6B). There was also a difference between the two pellets used in this experiment. The sperm solubilised with the 1DE LDS sample buffer was viscous due to the solubilisation of the DNA from sperm heads whereas sperm solubilised with the 2DE CHAPS was not viscous. Therefore, boar sperm solubilisation with the 1DE LDS sample buffer was more efficient than that with the 2DE CHAPS buffer.

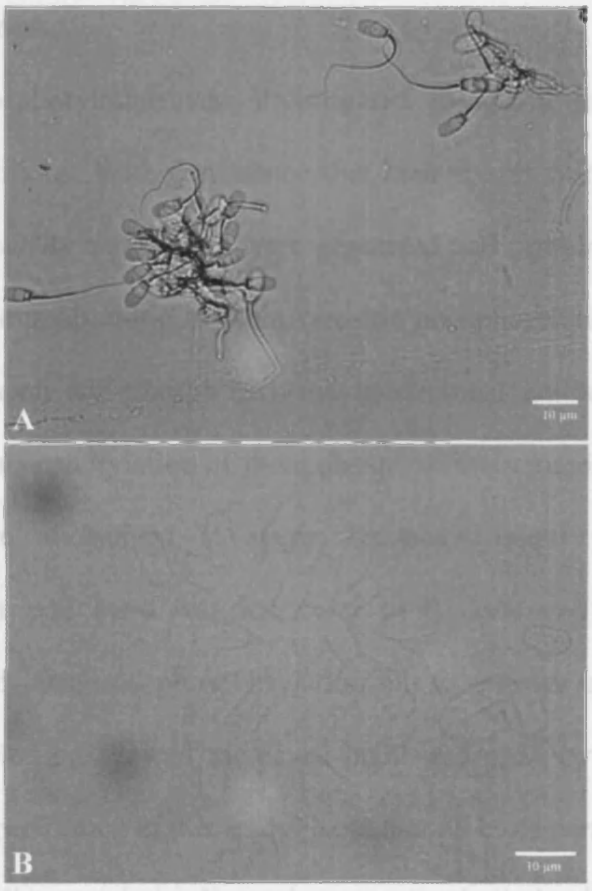

Figure 3.6: Boar sperm solubilised with 1DE LDS sample buffer were solubilised more than those with 2DE CHAPS buffer.

(A) Boar sperm was resuspended with the 2DE (urea/thiourea CHAPS) buffer lysed for 45 min at room temperature and centrifuged 10,000 *g* for 5 min. Sperm heads and tails are still intact and were completely solubilised. **(B)** Boar sperm was resuspended with 1DE LDS sample buffer, heated 70 °C and centrifuged. Sperm tails were completely solubilised and only head skeleton or nucleus remained.

3.5.5 Protein tyrosine phosphorylation is increased in sperm incubated under capacitating conditions

Tyrosine phosphorylation was investigated in sperm incubated under the experimental C conditions. With confidence that boar sperm were viable and motile under C conditions, whole cell lysates were generated and proteins were resolved by 1DE followed by immunoblotting. Protein tyrosine phosphorylation was assessed by immunoblotting with an anti-phosphotyrosine monoclonal antibody (4G10). Figure 3.7A shows protein phosphorylation of three phosphoprotein major bands pp45, pp40, and pp37 that were unchanged in sperm incubated under C conditions. The phosphorylation of a p32 band was increased in C compared with N sperm. In addition, p20 showed increased phosphorylation but to a lesser extent than the p32. Whilst the immunoblotting showed increased pp32 and pp20 by time, this was not observed in all blots performed in this study. Note that all blots performed in this study were assessed for similar protein loading using an anti-tubulin loading control. Tubulin protein was detected in all control samples at 50 kDa.

Indirect immunofluorescence localisation with the same antibody detected phosphorylated tyrosine in the acrosome and equatorial subsegment in N and C sperm incubated for 1 h (Figure 3.7B, a and b) while C sperm incubated for 3 h (Figure 3.7C) showed additional phosphorylation in the posterior ring, midpiece and principal piece which was not present in N sperm (data not shown but the same pattern as Figure 3.7Ba).

No immunofluorescence was observed in control slides (secondary antibody only) which convinced us that the fluorescence was due to the 4G10 Ab (Figure 3.8).

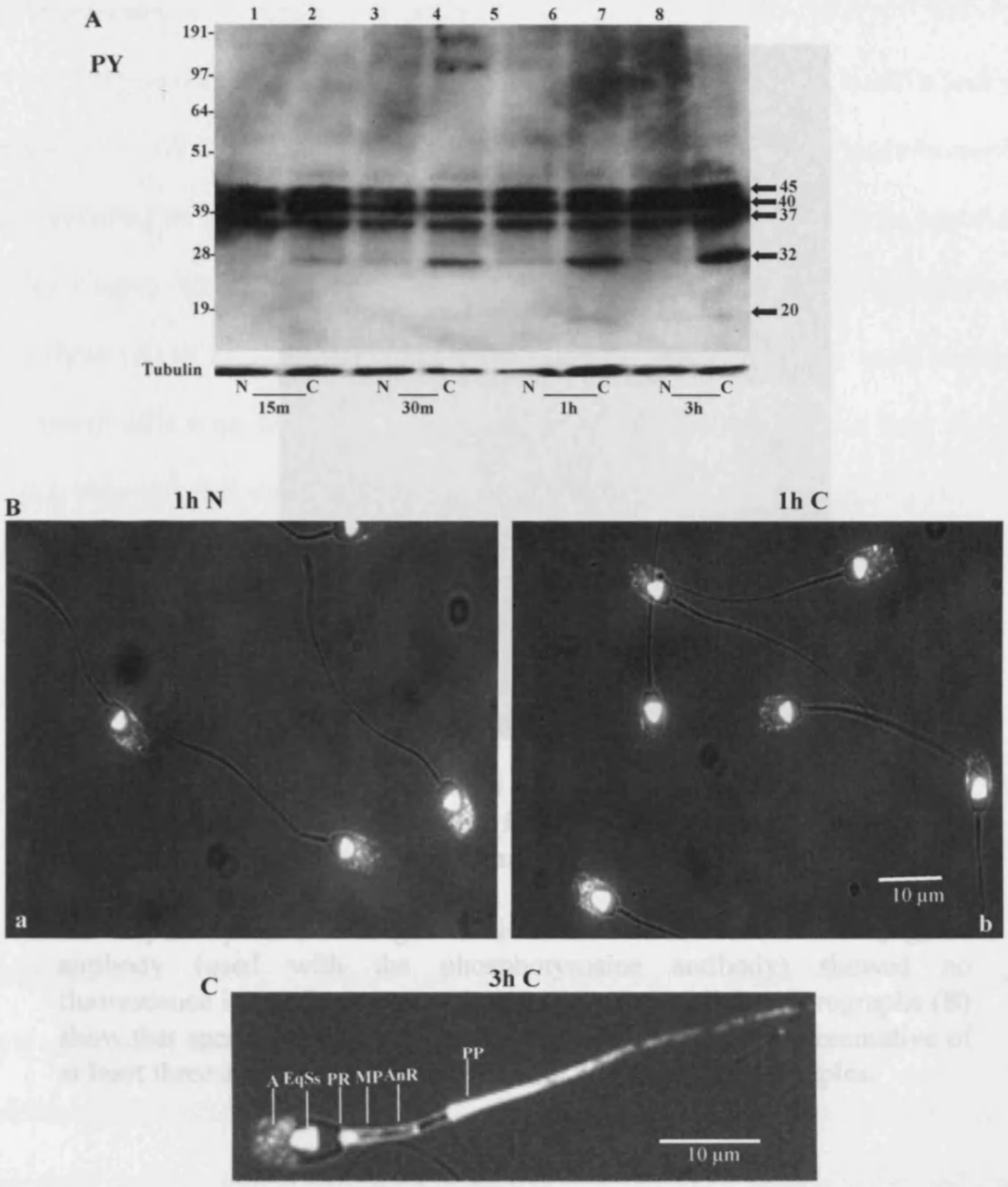

Figure 3.7: Protein tyrosine phosphorylation in sperm incubated under capacitating conditions (C) is similar to previously reported studies.

(A) Immunoblotting showing proteins detected with anti-phosphotyrosine antibody for sperm incubated under non-capacitating (N) and capacitating (C) conditions for 15 min, 30 min, 1 h and 3 h. **(B)** Immunolocalisation of phosphoproteins detected with the same antibody as panel A in fixed sperm cells after 1 h incubation in non-capacitating (N) or capacitating (C) conditions. **(C)** Immunolocalisation of phosphoproteins detected with the same antibody as panel A in fixed sperm cells after 3 h incubation in (C) conditions. paired images (fluorescence and light) are shown in **Ba, Bb** and **C.** Key: A, acrosome; EqSs, equatorial subsegment; PR, posterior ring; MP, midpiece; ApR, apical ridge; PP, AnR, annular ring; PP, principal piece. The results shown are representative of at least ten experiments performed with different sperm samples and tubulin loading controls were used for all blots.

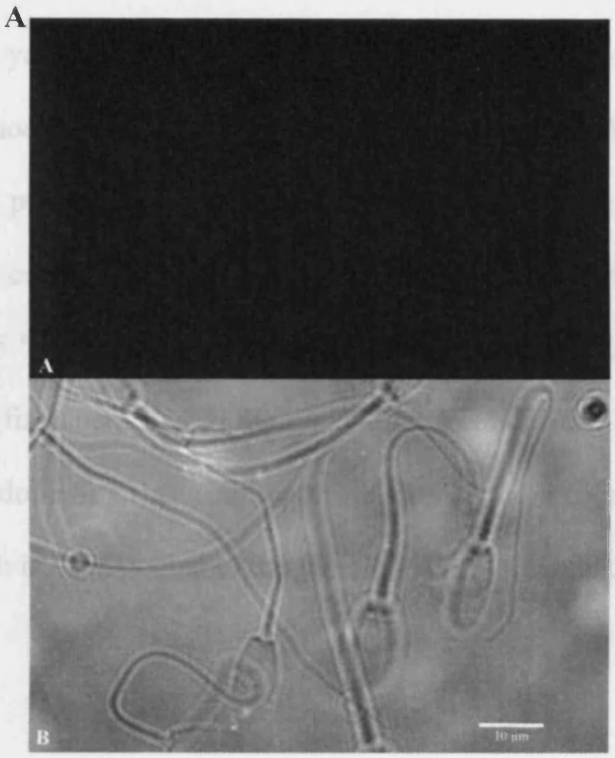

Figure 3.8: Control experiments using only anti-mouse secondary antibody demonstrates that the fluorescence observed for tyrosine phosphorylation is due to the primary antibody.

Boar sperm probed with goat anti-mouse Alexa Fluor 488-conjugated antibody (used with the phosphotyrosine antibody) showed no fluorescence in the fluorescence images **(A)**. Paired light micrographs **(B)** show that sperm cells are present. The results shown are representative of at least three experiments performed with different sperm samples.

3.6 Discussion

Despite the 60 years since the discovery of capacitation, there is still a lack of a reliable and easy method for assessing capacitation. This has resulted in an incomplete understanding of this process. Therefore, this chapter was undertaken to establish a model system. Sperm cells were incubated in Tyrode's medium with bicarbonate (C) or without (N) at 38.5 °C and 5% CO_2. It was very important in this study to ensure that sperm cells were first incubated in the media under conditions that keep the cells viable. This was first done by measuring the osmolality of the incubation buffer (295-298 mOsm / kg) which is nearly equal to the female tract (Fisch et al., 1990; Rossato et al., 1996).

Viability was the first assessment of the sperm incubated under N and C conditions. It was assessed to ensure that sperm cells used in this study are viable and able to undergo capacitation. Figure 3.3 shows that the number of viable sperm incubated for 1 h under C conditions was less than that incubated under N condition. This difference between N and C sperm viability was previously related to the combination of BSA and bicarbonate in C medium which the same medium used in this study and suggested that these both components may induce some cell death (Flesch et al., 1999).

Motility was also monitored to detect the changes after the incubation with and without bicarbonate. Capacitation confers upon the sperm an ability to gain hyperactivated motility, interact with oocyte zona pellucida (ZP), undergo the AR and initiate oocyte plasma membrane fusion (Yanagimachi, 1994). It was very important to

track sperm motility to detect the changes caused by bicarbonate on sperm incubated under C conditions. This motility was monitored manually under the light microscope during different time incubations. Before incubation after removing the extender buffer and washing the cells with TBSS, sperm motility was very slow and non progressive. Addition of incubation buffer which contains bicarbonate at 38.5 °C, sperm motility changed dramatically to be progressive due to the molecular changes for successful fertilisation (Figure 3.1A).

Hyperactivated mammalian sperm have been characterised by a vigorous and non-linear movement caused by increased amplitude of flagellar beats (Figure 3.1B). This form of motility has been observed in sperm at the site and the time of fertilisation and appears to be essential for fertilisation (Suarez and Ho, 2003). It has been suggested that the mechanical thrust due to hyperactivated motility is vital for sperm to penetrate the zona pellucida of the oocyte (Yanagimachi, 1969). In this study, a significant increase in motility was observed within 5 min of bicarbonate addition. This rapid increase of motility was previously reported in boar sperm (Harrison, 2004). Moreover, sperm motility changed from slow nonlinear to linear forward progression after 1 h incubation under C conditions. By 2 h of incubation under C condition, sperm motility became hyperactivated and reached a plateau. Interestingly, head to head agglutination was observed after 2 h and reached a plateau at 3 h of incubation under C conditions. This was previously reported by another group (Dr. Bart Gadella, Utrecht University, The Netherlands personal communication). Similar to this, head to head agglutination has been reported when boar sperm were incubated in a modified Krebs-Ringer HEPES medium with Ca^{2+} and HCO_3^- (Harayama et al., 1998). They have also

reported a head to head agglutination when boar sperm were incubated in a modified Krebs-Ringer HEPES medium with cAMP analog Sp-5, 6-dichloro-1-β-D-ribofuranosyl-benzimidazole-monophosphorothioate (cBiMPS) (Harayama, 2003). BSA was substituted in the incubation media with 0.1 % polyvinyl alcohol (PVA) because they have expected in a previous study that this component may stimulate the head to head agglutination (Harayama et al., 2000). They have concluded that the agglutination was due to the cAMP signalling which is connected to mobilisation of calcium from the putative acrosomal store to cytoplasm by the sperm head to head agglutination (Harayama et al., 2000) and subsequently increases early (S/T) phosphorylation and later tyrosine phosphorylation which both proteins localised in the flagellum (Harayama, 2003).

In contrast to C cells, N sperm investigated in this thesis showed no movement through the microscopic field in all experiments but instead demonstrated a vibrational movement ('static vibrance') caused by sperm sticking to the microscope slide due to a lower lateral head displacement than the C cells. This is consistent with the observation by other workers using the pig as a model system (Dr. Bart Gadella, Utrecht University, personal communication). To the best of my knowledge, there has been no previous report of motile sperm cells incubated under N conditions.

Acrosome integrity was also assessed after the incubation of sperm in N and C conditions before and after the different washing and centrifugation steps. These steps may damage and cause loss of acrosome integrity. Therefore, an immunofluorescence assay with 18.6 mAb was used to detect the acrosome integrity (Brewis et al., 1996).

This antibody is able to stain the acrosome and confirmed that there was no effect of these steps on acrosome integrity.

A previously reported increased tyrosine phosphorylation of pp32 in boar sperm was used to compare with the results in this study. Reports from several laboratories have documented that protein phosphorylation especially at tyrosine residues is one of the most important events that occur during capacitation. Studies have shown that increased phosphorylation of sperm proteins incubated under C conditions are an important aspect of capacitation and has been shown to be associated with hyperactivated motility (Si and Okuno, 1999), ZP binding (Burks et al., 1995; Urner et al., 2001) and the AR (Tardif et al., 2001).

Extensive research has started to elucidate various pathways involved in protein phosphorylation during sperm capacitation. Therefore, tyrosine phosphorylation was chosen as an indicator of the changes under capacitating conditions in this study. As mentioned in **Section 3.5.5**, protein tyrosine phosphorylation has been widely investigated and most studies demonstrated that proteins of boar sperm incubated under C conditions were more phosphorylated in tyrosine residues at pp32 and in one study a pp20 than with boar sperm incubated under N sperm (Bailey et al., 2005; Dube et al., 2003; Flesch et al., 1999; Harayama, 2003; Harayama and Nakamura, 2008; Harayama et al., 2004b; Kalab et al., 1998; Tardif et al., 2001). These results for tyrosine phosphorylation were consistent with the pp32 detected in this study. The pp32 protein is likely to be the same as the calcium-dependent phosphorylation during capacitation observed by (Bailey et al., 2005; Dube et al., 2003). On the other hand, the 20 kDa protein reported in this study has only been

previously reported by (Kalab et al., 1998). The pp32 protein tyrosine phosphorylation detected by (Tardif et al., 2001), was later analysed by the same research group (Bailey et al., 2005) to identify it. They demonstrated that the 32 kDa is not a single protein but it is composed of several tyrosine phosphoproteins. Unfortunately, that study was not able to conclusively identify this protein. These data demonstrated that sperm tyrosine phosphorylation changed over time in a similar fashion to that previously reported in boar sperm and convinced us that our system was suitable for the study of boar sperm capacitation *in vitro*.

Several studies have correlated the degree of tyrosine phosphorylation with the capacitative state of sperm. Visconti et al, examined the association between the capacitative state and protein tyrosine phosphorylation in mouse sperm (Visconti et al., 1995a). They observed a time dependent increase in the protein tyrosine phosphorylation of a set of specific proteins in the molecular range of pp 40–120, which was correlated with the capacitation state of sperm. This provides evidence that protein tyrosine phosphorylation is an important regulatory pathway in modulating the events associated with capacitation. However, in this study protein tyrosine phosphorylation was not found to be increased by the time of incubation under capacitating condition.

Sperm lysates were investigated in this study to assess whether complete lysis of head and tail structures occurred. The two different methods were chosen to solubilise sperm cells are 1DE LDS sample buffer and 2DE CHAPS buffer. The 1DE LDS sample buffer was found to be more efficient and completely solubulised the tails and most of the head structures. However, 2DE CHAPS buffer was unable to solubilise

the heads and the tails. Therefore 1DE LDS sample buffer is likely to be better than 2DE CHAPS for protein solubilisation and was chosen as the preferred method to extract boar sperm proteins in this study.

Overall it was confirmed in this chapter that the boar sperm model system developed is an excellent model system. Boar sperm proteins incubated under N and C conditions will be investigated in **Chapter 4** to characterise S/T protein phosphorylation.

CHAPTER 4

Bicarbonate-dependent serine/threonine (S/T) protein dephosphorylation in boar sperm

4.1 Introduction

Sperm capacitation is dependent on an early increase of intracellular cAMP levels from ATP by SACY and this activates PKA (de Lamirande et al., 1997; Lefievre et al., 2002; Olds-Clarke, 2003). This increase in cAMP and PKA activity regulates tyrosine phosphorylation which is strongly associated with the onset of sperm motility (Si and Okuno, 1999; Tash and Bracho, 1998; Visconti et al., 1995b). PKA may work through multiple pathways to regulate flagellum function, but one likely mechanism of its action is that S/T phosphorylation of PKA target proteins causes activation of downstream, yet unidentified, tyrosine kinase or kinases whose targets are primarily located in the flagellum (Leclerc et al., 1996; Visconti and Kopf, 1998).

The phosphorylation of a protein is a dynamic process controlled by both protein kinases and phosphatases (Johnson and Barford, 1993). Many of these kinases and phosphatases are specific for serine (S), threonine (T) or tyrosine (Y) residues and others have dual specificity (Hunter, 1991). S/T protein kinases such as PKA (Visconti and Kopf, 1998; Visconti et al., 1999a) and PKC (Harayama and Miyake, 2006) have been shown to regulate capacitation. However, little is known about the changes in protein S/T phosphorylation during capacitation. Early studies have suggested an association between PKA and protein tyrosine kinase/phosphatase pathways during capacitation (Visconti and Kopf, 1998). Thus, identifying proteins phosphorylated at S/T residues during capacitation may help to understand the molecular basis of capacitation. A few studies have reported increased S/T phosphorylation in sperm proteins incubated under capacitating conditions, including BSA, Ca^{2+} and HCO_3^- (Harrison, 2004; Jha and Shivaji, 2002b; Kaneto et al., 2008; O'Flaherty et al., 2004) or

with cAMP analogues (Harayama, 2003). Although these studies have used phospho (S/T) substrate antibodies that detect phospho (S/T) in the specific sequence recognised by PKA to identify PKA substrates during capacitation, the role of these PKA substrates needs to be clarified.

The aim of this chapter was to investigate S/T protein phosphorylation in sperm incubated under C conditions. We chose phosphokinase substrate antibodies that have been used to investigate other signalling systems but have not been widely used to study mammalian sperm proteins.

4.2 Aims

- Investigate bicarbonate-dependent changes in S/T protein phosphorylation in boar sperm incubated under C conditions
- Determine the effect of certain pharmacological inducers and inhibitors on phospho (S/T) proteins in sperm incubated under C conditions

4.3 Materials and methods

4.3.1 Sperm incubation with calyculin A and or dbcAMP/IBMX

In experiments where the influence of cAMP on (S/T) protein phosphorylation was evaluated, sperm were incubated for 1 h in N or C conditions in the absence or presence of 1 mM dibutyryl cAMP (dbcAMP, Sigma-Aldrich) which is a cAMP analogue and 100 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich) which is a phosphodiesterase inhibitor.

In experiments where the role of phosphatases on (S/T) protein phosphorylation was investigated cells were incubated for 1 h in N or C conditions in the absence or presence of either 100 or 250 nM calyculin A (Sigma-Aldrich), which is a pharmacological phosphatase inhibitor of PP2A and PP1 (IC_{50} of 0.5-1.0 nM and 2.0 nM, respectively) (Ishihara et al., 1989).

4.3.2 1DE and immunoblotting

See **Section 2.4 and 2.5** for 1DE and immunoblotting. The primary antibodies to detect S/T phosphoproteins were used at 1:1000 dilutions unless otherwise stated (Table 2.1). The secondary antibody used was goat anti-rabbit IgG (H+L) AP-conjugate (Bio-Rad Laboratories, Hemel Hempstead, UK) at 1:10,000 dilution. An α-tubulin antibody (was used as a loading control for each blot (50 kDa epitope) (Table 2.1).

4.3.3 Assessment of sperm motility in sperm incubated with calyculin A

Boar sperm were incubated for 1 h in C medium with or without calyculin A. Motility were recorded by manual inspection after 1 h under a bright-field microscope.

4.3.4 Addition of phosphatase inhibitors to the incubation buffers

Phosphatase Inhibitor Cocktail 1 (P2850, Sigma-Aldrich, UK) and Cocktail 2 (P5726, Sigma-Aldrich, UK) were both added 1 % (v/v). Control samples were N or C washed and lysed without phosphatase inhibitors. 'Washed' corresponds to samples where phosphatase inhibitors were added to the washing steps and 'lysed' corresponds to samples where phosphatase inhibitors were added during extraction prior to 1DE.

4.3.5 Indirect immunofluorescence (IIF)

For more details about IIF see **Section 2.6**. Slides were probed with primary antibodies at 1:100 dilution (Table 2.1). For each primary antibody used a control slide was processed and analysed where the primary antibody incubation was excluded and the cells were just incubated with secondary antibody. Slides were washed with PBS and mounted with Slow Fade Light antifade solution (Dako UK Ltd., Ely, UK). Slides were assessed by epifluorescence UV microscopy at 492 nm wave length with x 40 oil objective lens magnification to determine the localisation of detected phosphoproteins.

4.4 Results:

4.4.1 Loss of S/T protein phosphorylation in sperm incubated under capacitating conditions (C) was detected with polyclonal phospho Akt substrate antibody

Having established the conditions for *in vitro* sperm capacitation in **Chapter 3**, the changes in S/T protein phosphorylation were investigated using a range of phospho (S/T) kinase substrate antibodies. The first antibody tested was a phospho (S/T) Akt substrate antibody (Polyclonal Cat. #9611) (Alessi et al., 1996; Kane et al., 2002; Montminy, 1997). This commercial polyclonal antibody was produced by immunizing rabbits with phospho Akt substrate peptides and the resulting antibodies have been shown to have a specificity of R/K-X-R/K-X-X-T*/S* where X represents any amino acid. No cross-reactivity is observed with the corresponding nonphosphorylated sequences or with other phospho (S/T) containing motifs. The results in Figure 4.1A show distinct bands particularly in proteins above 50 kDa. While some bands did not change, we observed that pp97, pp96, pp90, pp64 and pp55 were reduced in C sperm when compared with N sperm throughout 1 h incubation (Figure 4.1A).

Immunolocalisation with this antibody detected phospho (S/T) protein in the flagellum (low levels), acrosome, equatorial subsegment, post-equatorial region and posterior ring in 1 h N sperm (Figure 4.1Ba). Less phosphorylation was present in 1 h C sperm (Figure 4.1Bb).

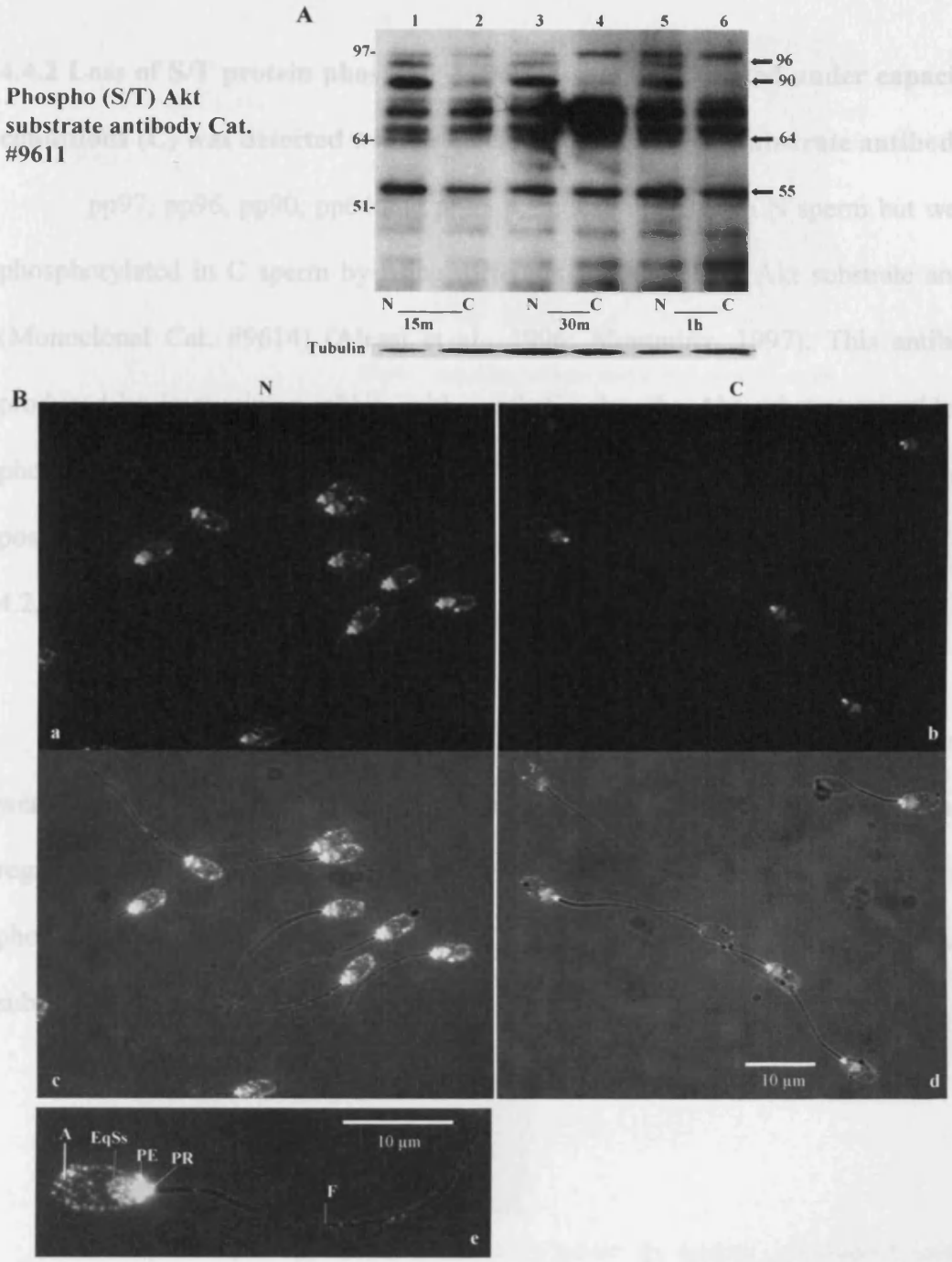

Figure 4.1: Loss of protein phosphorylation in sperm incubated under capacitating conditions (C) was detected with a polyclonal phospho Akt substrate antibody.

A polyclonal phospho-Akt substrate antibody was used to analyse the changes in protein phosphorylation using immunoblotting **(A)** and immunolocalisation **(B)** under non-capacitating (N) or capacitating (C) condition. The immunolocalisation results shown are following a 1 h incubation. **Ba** and **Bb** are fluorescence-only images, and paired images (fluorescence and light) are shown in **Bc** and **Bd,** respectively. **Be** represents an individual sperm cell from **Bc.** Key: A, indicates acrosome; EqSs, equatorial subsegment; PE, postequatorial region; PR, posterior ring; MP, midpiece; F, flagellum. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots.

4.4.2 Loss of S/T protein phosphorylation in sperm incubated under capacitating conditions (C) was detected with monoclonal phospho Akt substrate antibody

pp97, pp96, pp90, pp64 and pp55 were also detected in N sperm but were not phosphorylated in C sperm by using different phospho (S/T) Akt substrate antibody (Monoclonal Cat. #9614) (Alessi et al., 1996; Montminy, 1997). This antibody is produced by immunizing rabbits with synthetic phospho Akt substrate peptides. Akt phosphorylates substrates only at S/T in a conserved motif characterised by arginine at positions -5 and -3. This antibody recognises the motif R-X-R-X-X-T*/S* (Figure 4.2A).

Immunolocalisation with this antibody showed that phospho (S/T) proteins were localised in the apical ridge, equatorial subsegment (low levels), post equatorial region, posterior ring and flagellum (low levels) in 1 h N sperm (Figure 4.2Ba). No phosphorylation was observed in 1 h C sperm in the apical ridge and equatorial subsegment (Figure 4.2Bb).

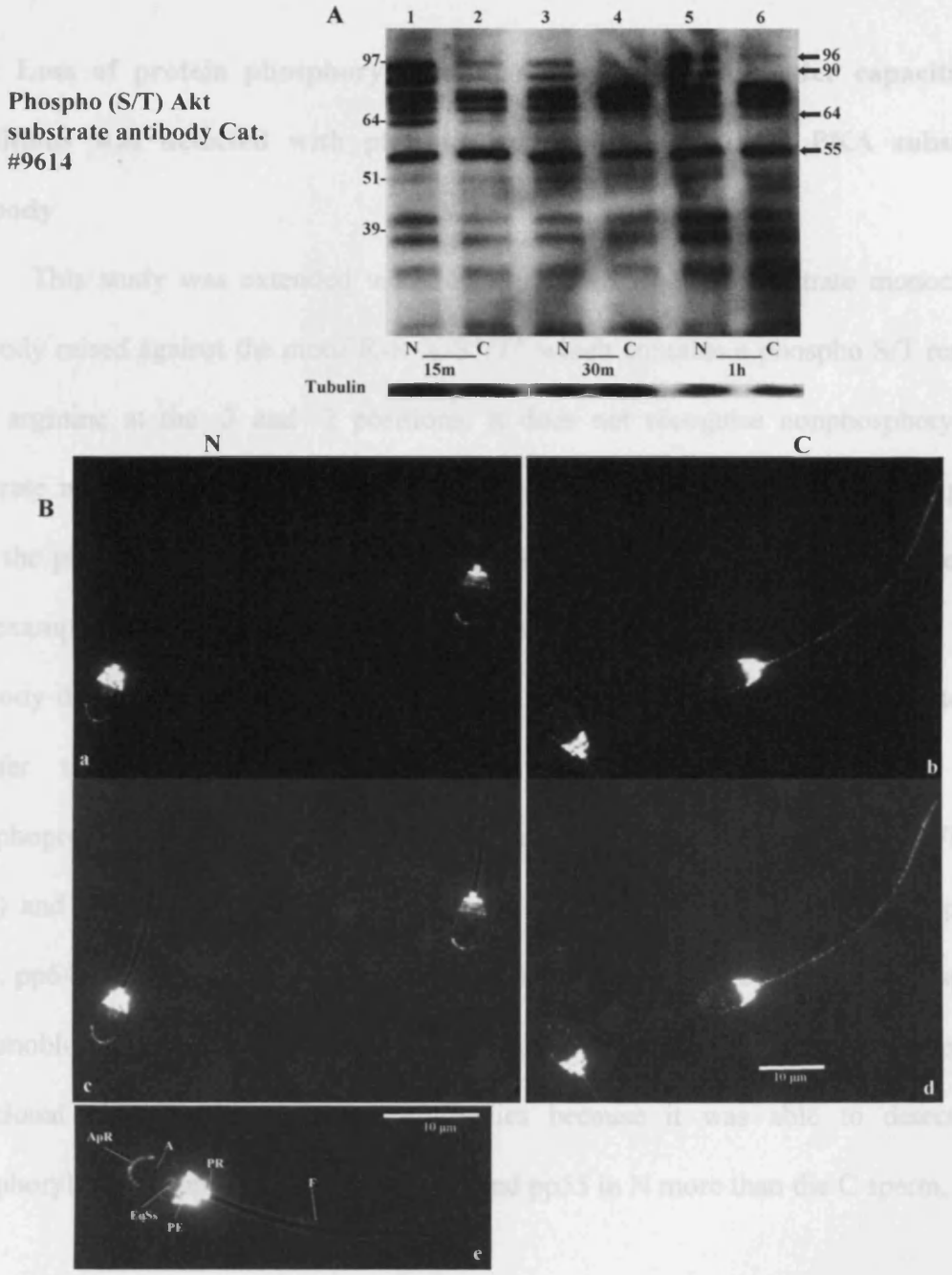

Figure 4.2: Loss of protein phosphorylation in sperm incubated under capacitating conditions (C) was detected with a monoclonal phospho Akt substrate antibody.

A monoclonal phospho Akt substrate antibody, was used to analyse the changes in protein phosphorylation using immunoblotting **(A)** and immunolocalisation **(B)** under non-capacitating (N) or capacitating (C) conditions. The immunolocalisation results shown are following a 1 h incubation. **Ba** and **Bb** are fluorescence-only images, and paired images (fluorescence and light) are shown in **Bc** and **Bd**, respectively. **Be,** represents an individual sperm cell from **Bc**. Key: A, indicates acrosome; EqSs, equatorial subsegment; PE, postequatorial region; PR, posterior ring; MP, midpiece; F, flagellum. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots

4.4.3 Loss of protein phosphorylation in sperm incubated under capacitating conditions was detected with phospho serine/threonine (S/T) PKA substrate antibody

This study was extended using a phospho (S/T) PKA substrate monoclonal antibody raised against the motif R-R-X-S*/T* which contains a phospho S/T residue with arginine at the -3 and -2 positions. It does not recognise nonphosphorylated substrate motif peptides. This antibody detected a wider range of proteins compared with the phospho Akt substrate antibody, particularly in the lower region of the gel. For example, it detected a pp35 band that was unchanged during capacitation. This antibody detected pp97 which some times did not appear in the blot may due to the transfer to the membrane. Interestingly, this antibody also detected five phosphoproteins in N sperm of the same size as those detected by the mono- (Figure 4.2A) and polyclonal (Figure 4.1A) phospho Akt substrate antibodies: pp97, pp96, pp90, pp64 and pp55 (Figure 4.3A). None of these proteins were detected in C sperm immunoblotting or IIF. The PKA substrate antibody was better than the mono and polyclonal phospho Akt substrate antibodies because it was able to detect the phosphorylation of pp97, pp96, pp90, pp64 and pp55 in N more than the C sperm.

Immunolocalisation with this antibody detected phosphoproteins in the flagellum (low levels), apical ridge, equatorial subsegment, and post-equatorial region in 1 h N sperm (Figure 4.3Ba). A similar pattern was observed in C sperm but less phosphorylation was present in the equatorial subsegment (Figure 4.3Bb).

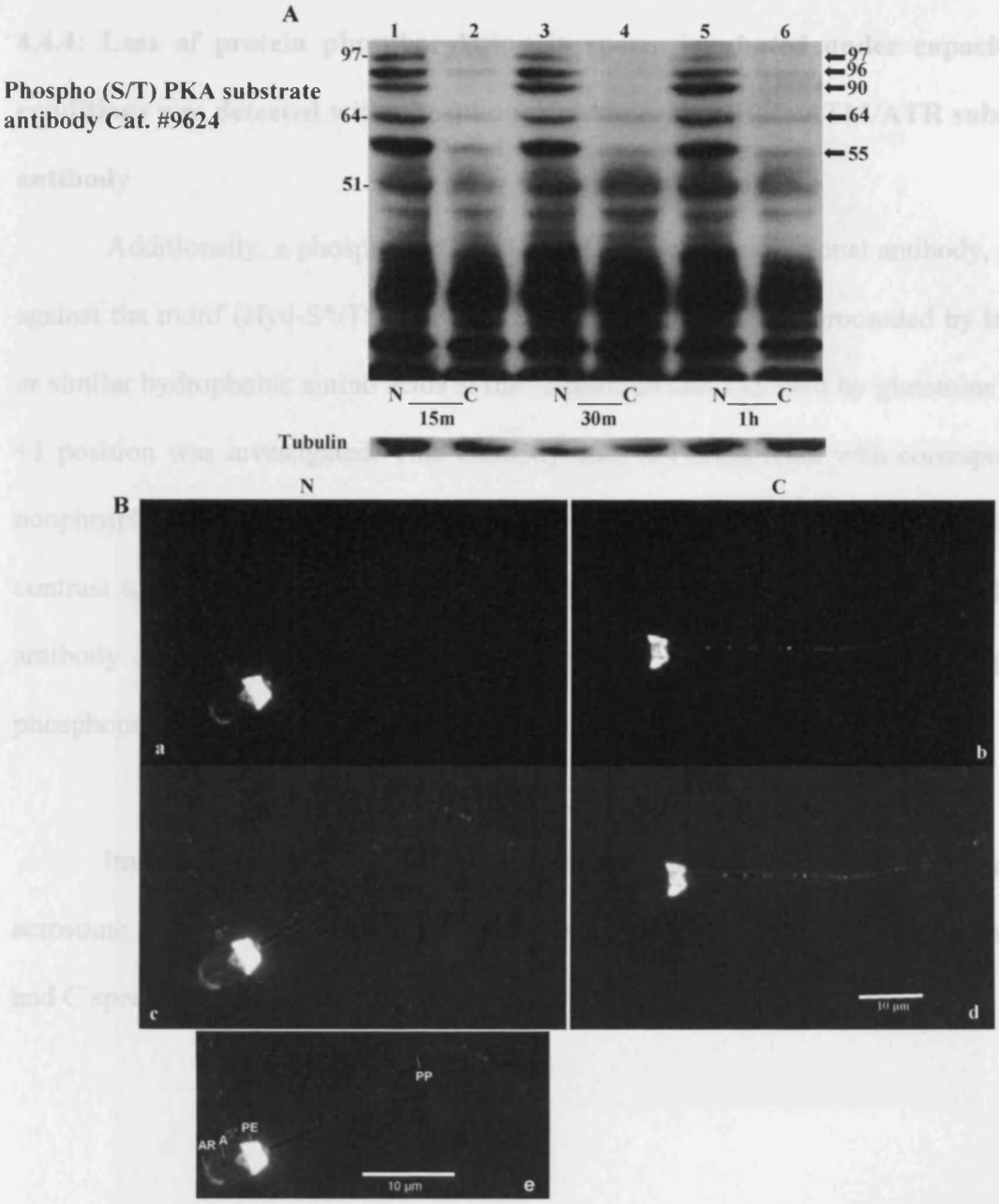

Figure 4.3: Loss of protein phosphorylation in sperm incubated under capacitating conditions (C) was detected with phospho serine/threonine (S/T) PKA substrate antibody.

Protein phosphorylation was analysed using a phospho serine/threonine (S/T) PKA substrate antibody. Immunoblotting **(A)** and immunolocalisation **(B)** data under non-capacitating (N) or capacitating (C) conditions are presented. The immunolocalisation results shown are following a 1 h incubation. **Ba** and **Bb** are fluorescence-only images and paired images (fluorescence and light) are shown in **Bc** and **Bd**, respectively. **Be** represents an individual sperm cell from **Bc**. Key: AR, indicates apical ridge; A indicates acrosome; PP, principal piece. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots.

4.4.4: Loss of protein phosphorylation in sperm incubated under capacitating conditions was detected with phospho serine/threonine (S/T) ATM/ATR substrate antibody

Additionally, a phospho (S/T) ATM/ATR substrate polyclonal antibody, raised against the motif (Hyd-S*/T*-Q) which contains phospho (S/T) proceeded by leucine or similar hydrophobic amino acids at the -1 position and followed by glutamine at the +1 position was investigated. This antibody does not cross-react with corresponding nonphosphorylated sequences or with other phospho (S/T) containing motifs. In contrast to the previous antibodies used in this study, the phospho (S/T) ATM/ATR antibody detected only three proteins. Interestingly, one of these proteins, a pp105 phosphoprotein was absent in C sperm compared with N sperm (Figure 4.4A).

Immunolocalisation with this antibody detected phosphoproteins in the acrosome, postequatorial region and flagellum (low levels) after 1 h incubation in N and C sperm (Figure 4.4Ba and b).

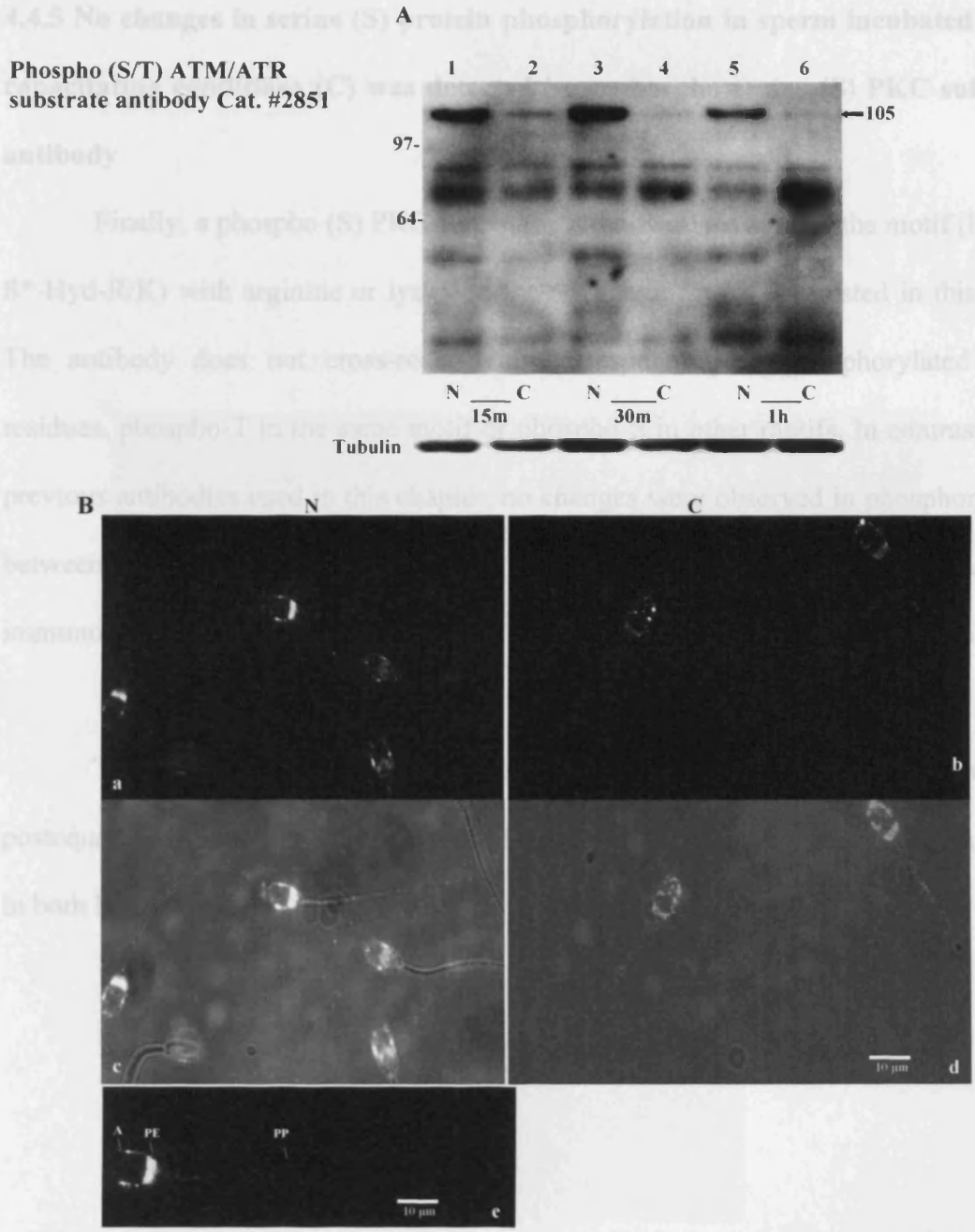

Figure 4.4: Loss of protein phosphorylation in sperm incubated under capacitating (C) conditions was detected with phospho serine/threonine (S/T) ATM/ATR substrate antibody.

Protein phosphorylation was analyzed using a phospho-serine/threonine (S/T) ATM/ATR substrate antibody. Immunoblotting **(A)** and immunolocalisation **(B)** data under non-capacitating (N) or capacitating (C) conditions are presented. The immunolocalisation results shown are following a 1 h incubation. **Ba** and **Bb** are fluorescence-only images and paired images (fluorescence and light) are shown in **Bc** and **Bd,** respectively. **Be** represents an individual sperm cell from **Bc**. Key: A, indicates acrosome; PE, postequatorial region;PP, principal piece. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. Key:

4.4.5 No changes in serine (S) protein phosphorylation in sperm incubated under capacitating conditions (C) was detected by a phospho serine (S) PKC substrate antibody

Finally, a phospho (S) PKC substrate antibody raised against the motif (R/K-X-S*-Hyd-R/K) with arginine or lysine at the +1 position was also tested in this study. The antibody does not cross-react with corresponding nonphosphorylated serine residues, phospho-T in the same motif or phospho-S in other motifs. In contrast to the previous antibodies used in this chapter, no changes were observed in phosphorylation between N and C sperm proteins by immunoblotting (Figure 4.5A) and immunofluorescence (Figure 4.5Ba and b) using phospho (S) PKC substrate antibody.

The immunofluorescence detected intensive fluorescence in the acrosome, postequatorial region, and midpiece, annular ring and principal piece of the flagellum in both N and C sperm (Figure 4.5Be).

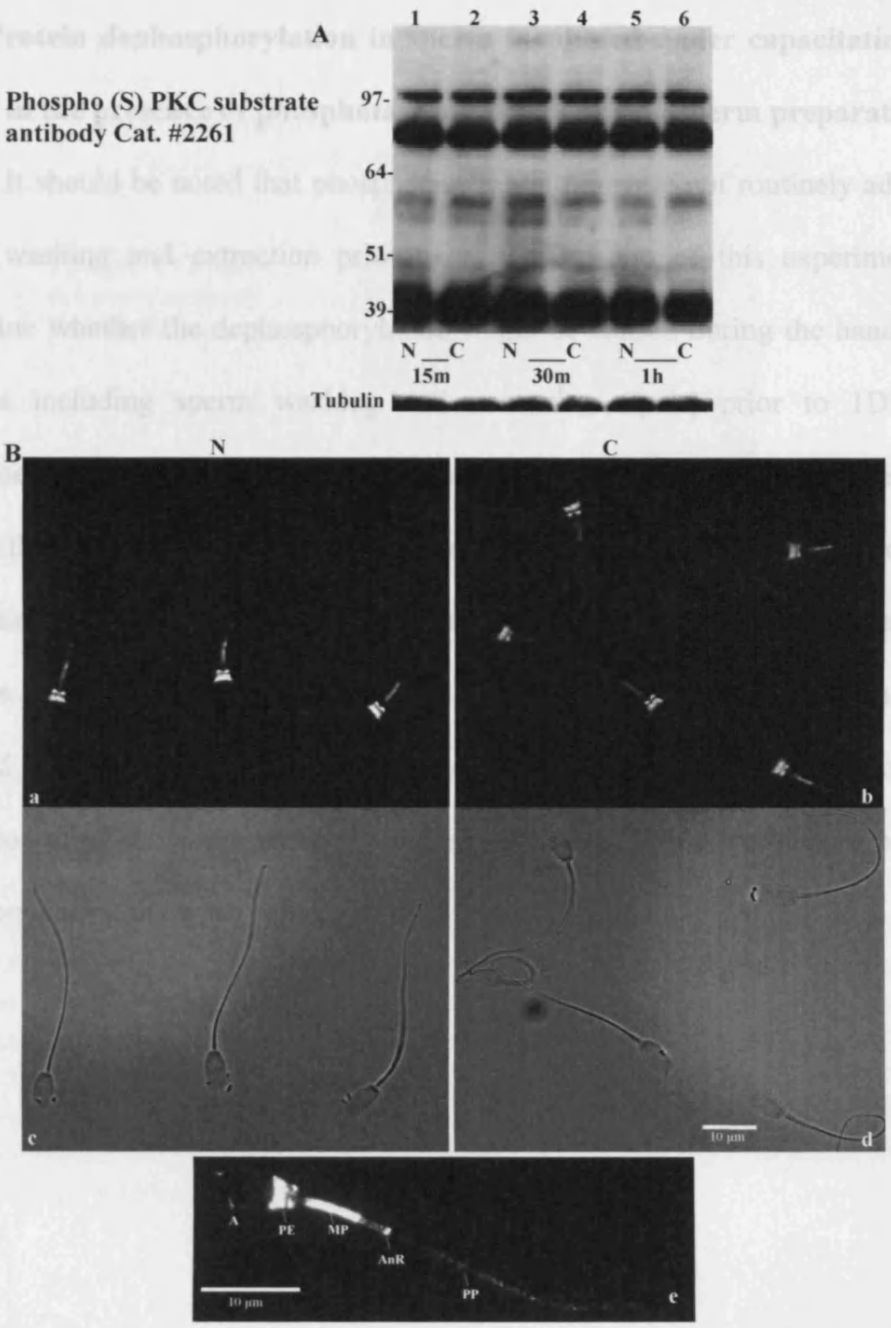

Figure 4.5: No changes in serine/threonine (S/T) protein phosphorylation dynamics in sperm incubated under capacitating (C) conditions using a phospho-serine/threonine (S/T) PKC substrate antibody.

Protein phosphorylation was analyzed using a phospho-serine/threonine (S/T) PKC substrate antibody motif. Immunoblotting **(A)** and immunolocalisation **(B)** data under non-capacitating (N) or capacitating (C) conditions are presented. The immunolocalisation results shown are following a 1 h incubation. **Ba** and **Bb** are fluorescence-only images and paired images (fluorescence and light) are shown in **Bc** and **Bd**, respectively. **Be** represents an individual sperm cell from **Bc** The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; PE, postequatorial region; MP, midpiece, AnR, annular ring; PP, principal piece.

4.4.6 Protein dephosphorylation in sperm incubated under capacitating (C) still occurs in the presence of phosphatase inhibitors during sperm preparation

It should be noted that phosphatase inhibitors were not routinely added during sperm washing and extraction prior to 1DE. The aim of this experiment was to determine whether the dephosphorylation might be caused during the handling of the samples including sperm washing and extraction (lysis) prior to 1DE. Control experiments were carried out using a broad range phosphatase inhibitor cocktail added to both the wash and extraction buffer immediately prior to 1DE. Sperm proteins were then transferred to a membrane and immunoblotted with PKA substrate Ab. The addition of the phosphatase inhibitors did not increase the phosphorylation in washed or lysed comparing with control samples (Figure 4.6). Note that dephosphorylation of the major epitopes is seen in all of the C samples even in the presence of phosphatase inhibitors during the wash or lysis steps.

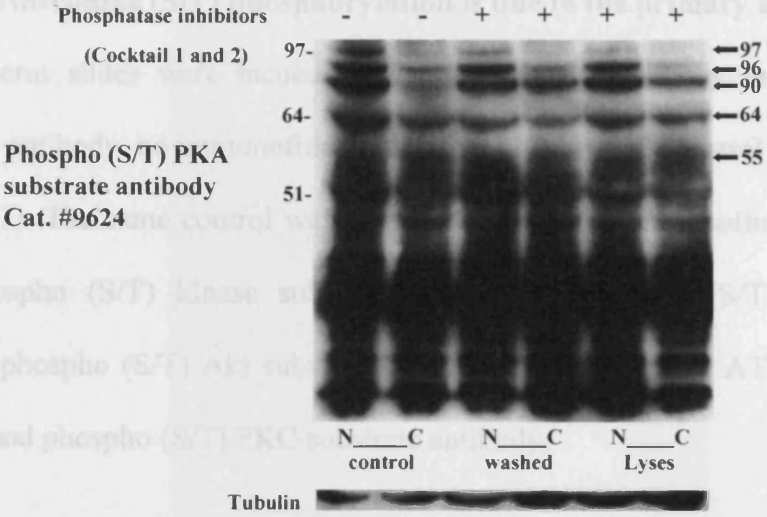

Figure 4.6: Protein dephosphorylation of capacitating (C) sperm still occurs in the presence of phosphatase inhibitors during sperm preparation for one dimensional electrophoresis (1DE).

Phosphatase Inhibitor Cocktail 1 and Cocktail 2 were both added (1 % (v/v)). Control samples were incubated for 1 h under non-capacitating (N) or capacitating (C) conditions washed and lysed without phosphatase inhibitors. 'Washed' corresponds to samples where phosphatase inhibitors were added to the washing steps and 'lysed' corresponds to samples where phosphatase inhibitors were added during extraction prior to 1DE. Samples were blotted using a phospho (S/T) PKA substrate antibody. Tubulin was used as a loading control. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots.

4.4.7 Anti-rabbit secondary antibody demonstrates that the fluorescence observed for serine/threonine (S/T) phosphorylation is due to the primary antibody only

Sperm slides were incubated without primary antibody and only with the secondary antibody, no immunofluorescence was observed in control slides in all cases (Figure 4.7). The same control was employed for all the immunofluorescence studies using phospho (S/T) kinase substrate antibodies (phospho (S/T) PKA substrate antibody, phospho (S/T) Akt substrate antibody, phospho (S/T) ATM/ATR substrate antibody and phospho (S/T) PKC substrate antibody.

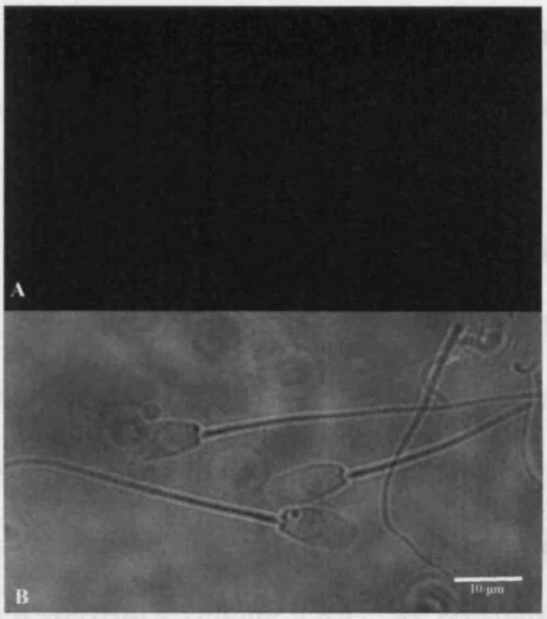

Figure 4.7: Control experiments using only anti-rabbit secondary antibody demonstrates that the fluorescence observed for serin/threonine (S/T) phosphorylation is due to the primary antibody

Boar sperm probed with goat anti-rabbit Alexa Fluor 488-conjugated antibody (used with the phospho-(S/T) kinase substrate antibodies) showed no fluorescence in the fluorescence images **(A)**. Paired light micrographs **(B)** show that sperm cells are present. The results shown are representative of at least 3 experiments performed with different sperm samples.

4.4.8: Dibutyryl cAMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX) treatment is not sufficient to cause serine/threonine (S/T) protein dephosphorylation under capacitating (C) conditions detected with a phospho serine/threonine (S/T) Akt substrate polyclonal antibody

To determine the effect of cAMP on S/T protein phosphorylation during capacitation, washed sperm were incubated with dbcAMP/IBMX under N and C conditions for 1 h. Cell extracts were generated and proteins were separated by 1DE followed by immunoblotting. S/T protein phosphorylation was first detected with the Akt substrate polyclonal antibody. No loss of phosphorylation was detected in N sperm in the presence of dbcAMP/IBMX (Figure 4.8A). As previously observed in Figure 4.1A, the pp96, pp90, pp64 and pp55 were absent in C compared with N sperm. Although the immunoblotting Figure 4.1A was probed with the same phospho Akt substrate polyclonal antibody in Figure 4.8A, it was notable that the former differ in that more bands were detected with darker background. This difference which may be due to the transfer conditions did not affect the results shown in both figures. Interestingly, additional phosphoproteins (for example, bands were observed at pp68, pp51 and pp29) were observed in C sperm in the presence of dbcAMP/IBMX compared with C sperm incubated in the absence of dbcAMP/IBMX or N sperm.

Phospho (S/T) proteins detected by this antibody were localised in the acrosome (low levels), equatorial subsegment and postequatorial region in N and C sperm incubated in the absence of dbcAMP/IBMX (Figure 4.8Ba and b). Immunolocalisation of this antibody was in similar cellular regions in N cells in the presence of dbcAMP/IBMX (Figure 4.8Bf). The phosphorylation was enhanced in the

postequatorial region, posterior ring and midpiece following 1 h incubation with dbcAMP/IBMX in C sperm (Figure 4.8Bg).

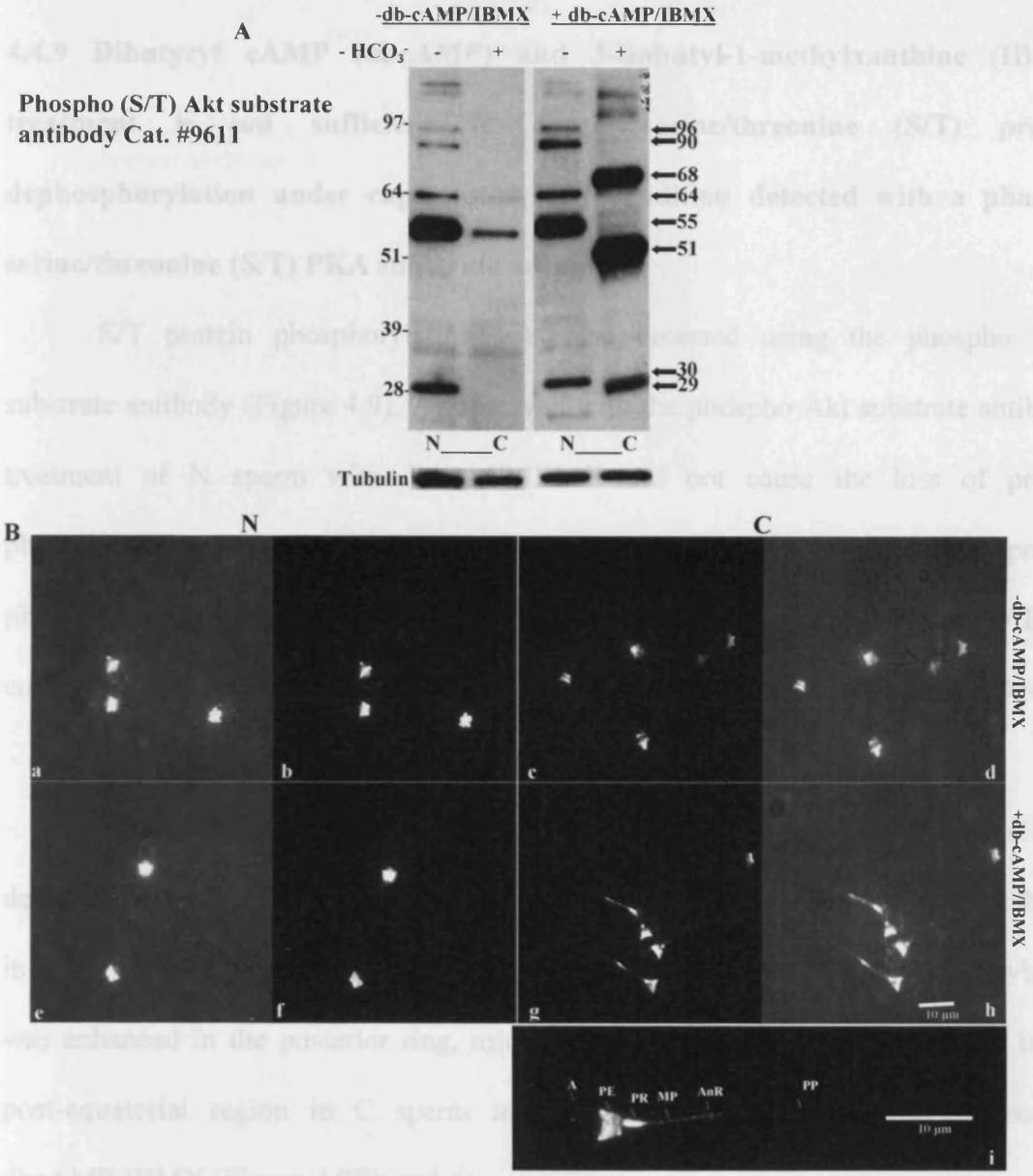

Figure 4.8: The addition of dibutyryl cAMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX) is not sufficient to cause serine/threonine (S/T) protein dephosphorylation under capacitating (C) conditions detected with a phospho serine/threonine (S/T) Akt substrate polyclonal antibody.

Cells were treated with dbcAMP/IBMX for 1 h under both non-capacitating (N) and capacitating (C) conditions. Subsequently they were analysed by immunoblotting (**A**) and immunolocalisation (**B**) as described. **Bb, Bc, Bf** and **Bg** are fluorescence only images and paired images (fluorescence and light) are shown in **Ba, Bd, Be** and **Bh,** respectively. **Bi**, represents an individual sperm cell from **Bg**. Key: A indicates, acrosome; PE, post-equatorial region; PR, posterior ring; MP, midpiece; AnR, annular ring; PP principal piece. The results shown are representative of at least three experiments performed with different sperm samples and tubulin loading controls were used for all blots.

4.4.9 Dibutyryl cAMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX) treatment is not sufficient to cause serine/threonine (S/T) protein dephosphorylation under capacitating (C) condition detected with a phospho serine/threonine (S/T) PKA substrate antibody

S/T protein phosphorylation was also detected using the phospho PKA substrate antibody (Figure 4.9). As observed with the phospho Akt substrate antibody, treatment of N sperm with dbcAMP/IBMX did not cause the loss of protein phosphorylation which was observed in C sperm. Again enhanced protein phosphorylation was observed in C sperm in the presence of dbcAMP/IBMX compared with C sperm incubated without dbcAMP/IBMX or N sperm (Figure 4.9A).

Immunofluorescence analysis with phospho (S/T) PKA substrate antibody detected phosphoproteins localised in the post-equatorial region in N sperm incubated in the presence or absence of dbcAMP/IBMX (Figure 4.9Ba and c). Phosphorylation was enhanced in the posterior ring, midpiece and principal piece but reduced in the post-equatorial region in C sperm in the presence but not in the absence of dbcAMP/IBMX (Figure 4.9Bb and d).

Furthermore, when sperm were incubated with dbcAMP/IBMX, no changes were observed on the pp105 protein detected by phospho (S/T) ATM/ATR substrate antibody in N cells but not in C cells (Figure 4.10).

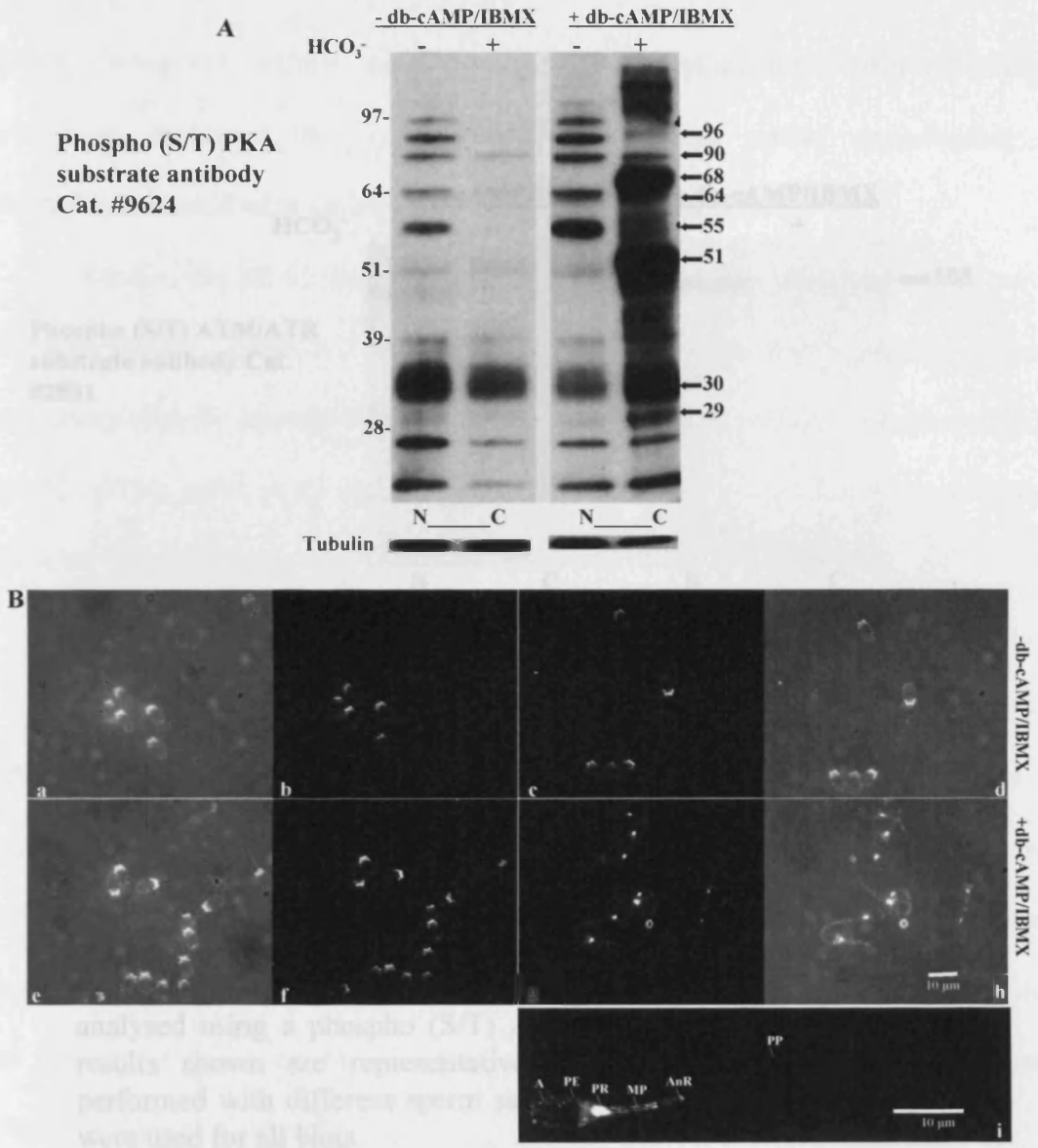

Figure 4.9: The addition of dibutyryl cAMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX) is not sufficient to cause serine/threonine (S/T) protein dephosphorylation under capacitating (C) conditions detected with a phospho serine/threonine (S/T) PKA substrate antibody.

Cells were treated with db-cAMP/IBMX for 1 h under both non-capacitating (N) and capacitating (C) conditions. Subsequently they were analysed by immunoblotting **(A)** and immunolocalisation **(B)** as described. **Bb, Bc, Bf** and **Bg** are fluorescence only images and paired images (fluorescence and light) are shown in **Ba, Bd, Be and Bh**, respectively. **Bi** represent an individual sperm cell from **Bg**. Key: A indicates, acrosome; PE, post-equatorial region; PR, posterior ring; MP, midpiece; AnR, annular ring. The results shown are representative of at least three experiments performed with different sperm samples and tubulin loading controls were used for all blots.

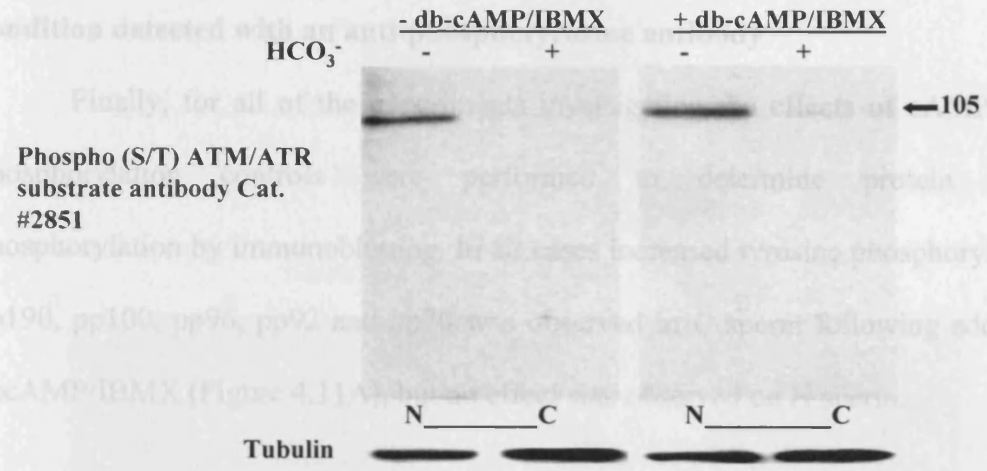

Figure 4.10: The addition of dibutyryl cAMP and 3-isobutyl-1-methylxanthine (dbcAMP/IBMX) is not sufficient to cause S/T protein dephosphorylation under capacitating (C) conditions detected with a phospho serine/threonine (S/T) ATM/ATR substrate antibody.

Cells were treated with db-cAMP/IBMX for 1 h under both non-capacitating (N) and capacitating (C) conditions. Subsequently they were analysed by immunoblotting and protein phosphorylation was analysed using a phospho (S/T) ATM/ATR substrate antibody. The results shown are representative of at least three experiments performed with different sperm samples and tubulin loading controls were used for all blots.

4.4.10 Dibutyryl cAMP and 3-isobutyl-1-methylxanthine (dbcAMP/IBMX) treatment increased the tyrosine phosphorylation under capacitating (C) condition detected with an anti-phosphotyrosine antibody

Finally, for all of the experiments investigating the effects of cAMP on S/T phosphorylation controls were performed to determine protein tyrosine phosphorylation by immunoblotting. In all cases increased tyrosine phosphorylation of pp190, pp100, pp96, pp92 and pp70 was observed in C sperm following addition of dbcAMP/IBMX (Figure 4.11A), but no effect was observed on N sperm.

Moreover, immunolocalisation with the same antibody detected phosphorylated tyrosine in the acrosome and equatorial subsegment in N (Figure 4.11Ba) and few sperm showed extra phosphorylation in principal piece of sperm tail of C sperm incubated without dbcAMP/IBMX for 1 h (Figure 4.11Bb) while all C sperm incubated with dbcAMP/IBMX (Figure 4.11Bd) showed additional phosphorylation in the posterior ring, midpiece and principal piece which was not present in N sperm (Figure 4.11Bc).

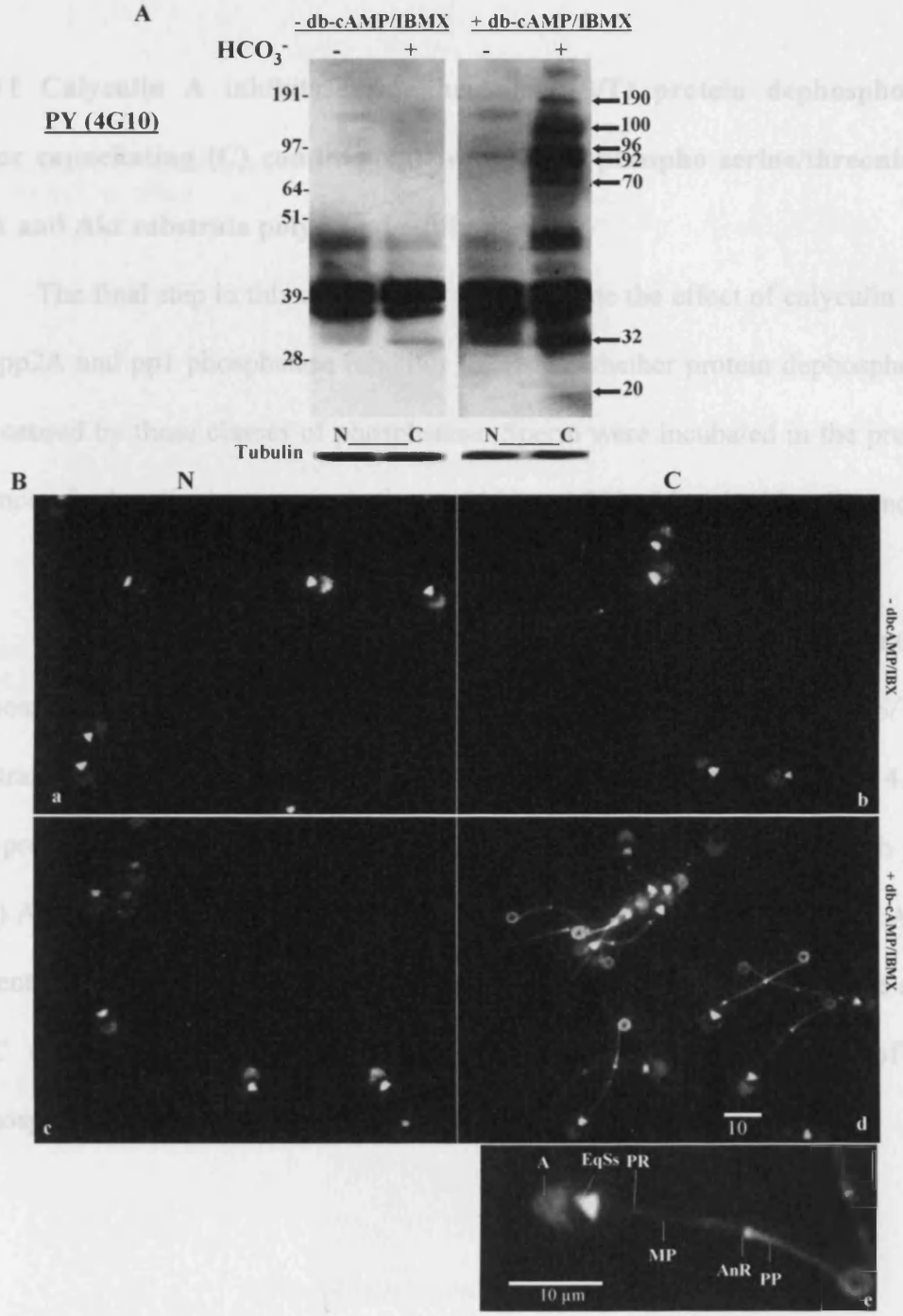

Figure 4.11: The addition of dibutyryl cAMP and 3-isobutyl-1-methylxanthine (dbcAMP/IBMX) increased the tyrosine phosphorylation under capacitating (C) condition detected with an anti-phosphotyrosine.

Cells were treated with dbcAMP/IBMX for 1 h under both non-capacitating (N) and capacitating (C) conditions. Subsequently they were analysed by immunoblotting (**A**) and immunolocalisation (**B**) as described. The immunolocalisation results shown are following a 1 h incubation. **Ba** and **Bc** without dbcAMP/IBMX and **Bb** and **Bd,** with dbcAMP/IBMX. **Ba, Bb, Bc** and **Bd** are paired images (fluorescence and light). **Be** represents an individual sperm cell from **Bd. Key:** A indicates acrosome; EqSs, equatorial subsegment; MP, midpiece, AnR, annular ring; PP, principal piece. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots.

4.4.11 Calyculin A inhibits serine/threonine (S/T) protein dephosphorylation under capacitating (C) conditions detected with phospho serine/threonine (S/T) PKA and Akt substrate polyclonal antibodies

The final step in this chapter was to investigate the effect of calyculin A which is a pp2A and pp1 phosphatase inhibitor to assess whether protein dephosphorylation was caused by these classes of phosphatase. Sperm were incubated in the presence or absence of calyculin A at concentrations of 100 and 250 nM under N or C conditions.

Immunoblotting results showed that calyculin A prevented the dephosphorylation of five protein bands detected with the phospho (S/T) PKA substrate antibody in C sperm (pp97, pp96, pp90, pp64 and pp55) (Figure 4.12A). It also prevented the dephosphorylation of similar protein bands detected with phospho (S/T) Akt substrate polyclonal antibody (Figure 4.12B). However, calyculin A did not prevent the loss of the pp105 detected by phospho (S/T) ATM/ATR substrate antibody in C cells (Figure 4.12C). This suggests two distinct mechanisms of protein dephosphorylation in boar sperm incubated under C conditions.

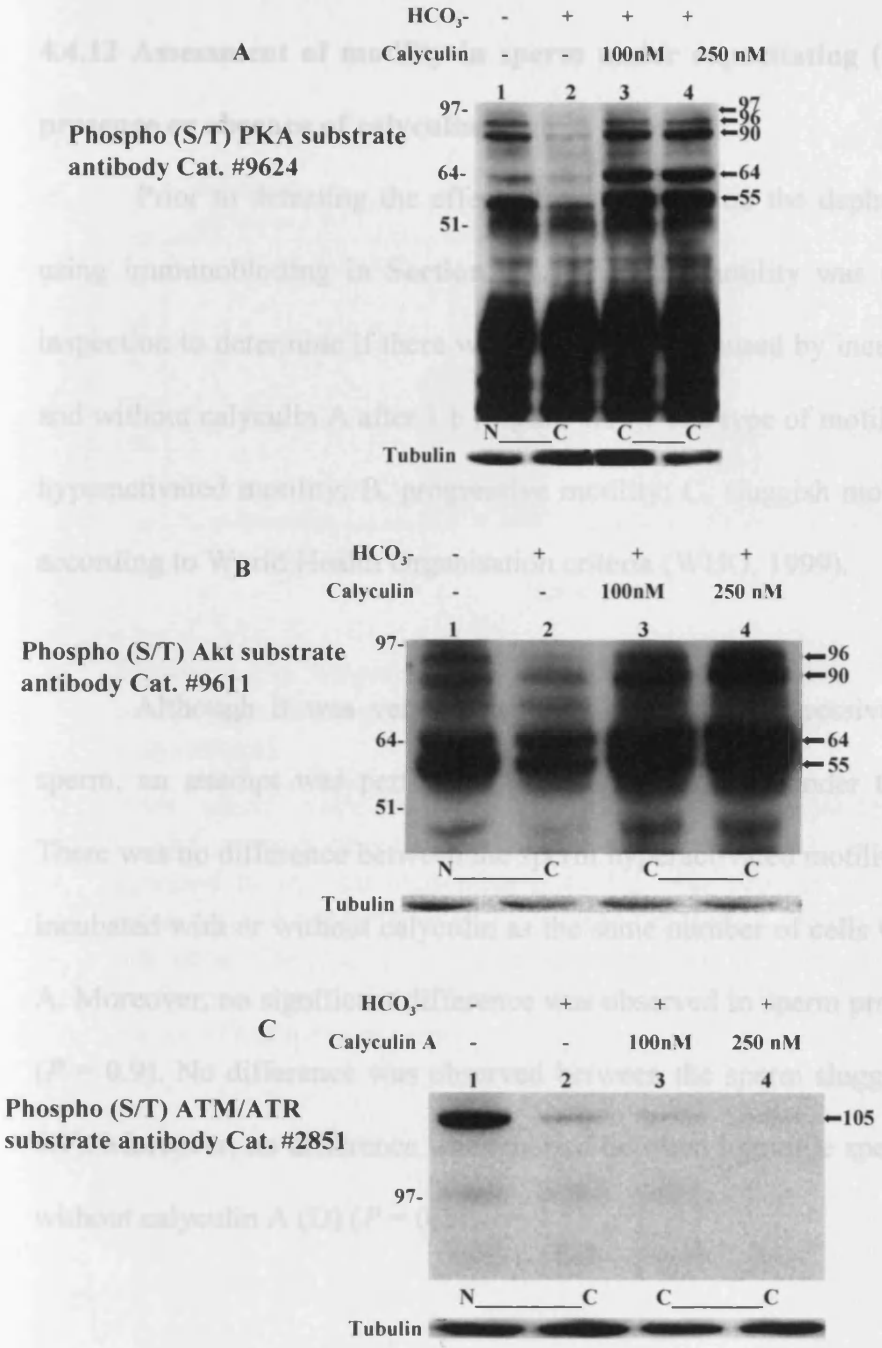

Figure 4.12: Calyculin A inhibits serine/threonine (S/T) protein dephosphorylation incubated under capacitating (C) conditions detected with phospho serine/threonine (S/T) PKA and Akt substrate polyclonal antibodies but not when detected with phospho serine/threonine (S/T) ATM/ATR substrate antibody.

Cells were treated with two concentrations of calyculin A for 1 h, as indicated under both non-capacitating (N) and capacitating (C) conditions. Subsequently protein phosphorylation was analysed by immunoblotting using a phospho (S/T) ATM/ATR substrate antibody **(A)** a phospho (S/T) PKA substrate antibody **(B)** and a phospho (S/T) Akt substrate polyclonal antibody **(C).** The results shown are representative of at least three experiments performed with different sperm samples and tubulin loading controls were used for all blots.

4.4.12 Assessment of motility in sperm under capacitating (C) conditions in the presence or absence of calyculin A

Prior to detecting the effect of calyculin A on the dephosphorylated proteins using immunoblotting in **Section 4.4.11**, sperm motility was examined by manual inspection to determine if there were any changes caused by incubation of sperm with and without calyculin A after 1 h (Figure 4.13). The type of motility was referred to A, hyperactivated motility; B, progressive motility; C, sluggish motility and D immotile according to World Health Organisation criteria (WHO, 1999).

Although it was very difficult to count the progressive and hyperactivated sperm, an attempt was performed to count these cells under the light microscopy. There was no difference between the sperm hyperactivated motility (A) between sperm incubated with or without calyculin as the same number of cells were counted in both. A. Moreover, no significant difference was observed in sperm progressive motility (B) (P = 0.9). No difference was observed between the sperm sluggish motility (C) (P = 0.9). Moreover, no difference was reported between immotile sperm incubated with or without calyculin A (D) (P = 0.2).

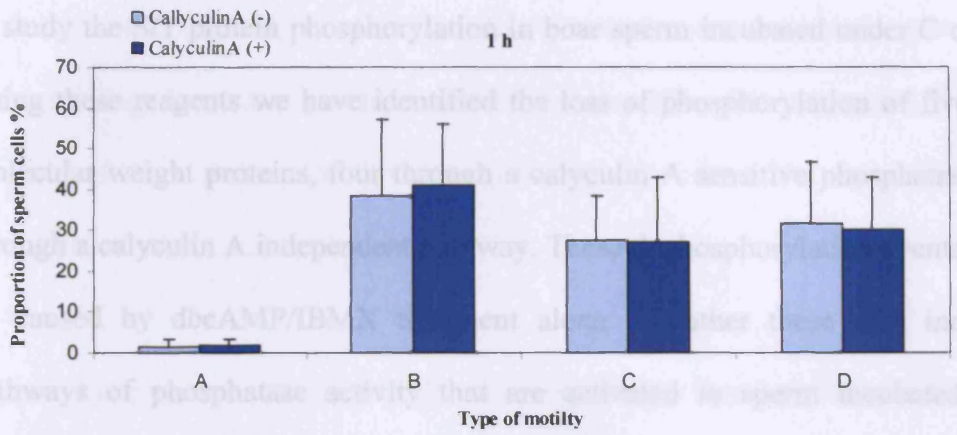

Figure 4.13: Assessment of motility in sperm cells incubated under capacitating (C) conditions in the presence and absence of calyculin A.

Boar sperm were washed and incubated in capacitating (C) conditions with or without calyculin A (Calyculin A). Motility was assessed by manual inspection after 1 h. The type of motility was referred to A, hyperactivated ; B, progressive; C, sluggish and D immotile. The results shown are representative of three experiments performed with different sperm samples. The data presented is the mean of three experiments.

4.5 Discussion

In this chapter, phosphokinase substrate antibodies were used for the first time to study the S/T protein phosphorylation in boar sperm incubated under C conditions. Using these reagents we have identified the loss of phosphorylation of five different molecular weight proteins, four through a calyculin A sensitive phosphatase and one through a calyculin A independent pathway. These dephosphorylation events could not be caused by dbcAMP/IBMX treatment alone. Together these data indicate two pathways of phosphatase activity that are activated in sperm incubated under C downstream of the bicarbonate sensor in a cAMP-independent manner.

We deliberately chose to study S/T phosphorylation using four antibodies that have not been used previously in boar sperm, including phospho (S/T) Akt substrate (monoclonal and polyclonal), phospho (S/T) ATM/ATR and PKC substrate antibodies. With the phospho (S/T) Akt substrate antibodies, we observed dephosphorylation of five proteins in sperm incubated under C conditions while increased phosphorylation of other proteins was observed following treatment with dbcAMP/IBMX. These changes contrasted with the phospho (S/T) PKC substrate antibody which detected similar proteins under all experimental conditions.

S/T protein dephosphorylation was detected with a PKA substrate antibody that was chosen as a comparison because it had been used in other studies (Harayama and Miyake, 2006; Harayama and Nakamura, 2008; Harrison, 2004; Kaneto et al., 2008; O'Flaherty et al., 2004). One previous study has reported decreased phosphorylation of a single 100 kDa on S/T protein during capacitation using a different anti-phospho (S)

antibody in the hamster (Jha and Shivaji, 2002). However, the majority of studies have focused on increases in phosphorylation. Thus, to my knowledge, this study is the first to document multiple dephosphorylation events that occur in a bicarbonate-dependent fashion. Together, this provides direct evidence for more complex S/T protein phosphorylation dynamics than is generally described for sperm undergoing capacitation.

There was an overlap with the sizes of proteins detected with the Akt substrate antibodies and the PKA substrate antibodies, particularly in N conditions. Despite the overlap, when comparing the Akt substrate antibodies and the PKA substrate antibody using immunofluorescence, the pictures look subtly different, probably due to the detection of other proteins by the antibodies. Although different motifs of S/T PKA substrate antibody have been used in this study than that previously used by others (on boar sperm), the immunolocalisation of these phosphoproteins was similar (Adachi et al., 2008; Harayama, 2003). Interestingly, phosphorylation seemed to be lost in the head region with the Akt substrate monoclonal antibody (Figure 4.2Bb) which may suggest a role in zona binding. There is a similarity between the motifs recognised by the Akt and PKA substrate antibodies: the R-R-X-S*/T* motif of the PKA substrate would be included within the R-X-R-X-X-S*/T* of the Akt substrate antibody. Given this, it would be predicted that pp97, pp96, pp90, pp64 and pp55 contain a motif that is made up of both sequences, perhaps R-X-R-R-X-S*/T*.

On a more technical note the diference between phosphorylated and dephosphorylated proteins in both N and C sperm was detected by visually comparing

the intensity of protein bands produced by immunoblotting. This was appropriate in most experiments as the differences were obvious (either a marked difference or presence/absence). However, on reflection it may have been wise to perform densitometric analysis of mutliple blots. This may have been beneficial in situations where there were not clear visible differences between N and C sperm. One example of this is the 55 kDa phosphorylation intensity in Figure 4.2A. 112. The immunolocalisation results revealed clear predominant patterns of phosphorylation (at least 80% of cells) and the typical pattern was presented. However, again on reflection, it may have been prudent to quantify this more carefully by counting predominant patterns or assessing fluorecence intensity.

These experiments show that S/T dephosphorylation across all six proteins studied occurs within 15 min of incubation under capacitating conditions. This rapid response indicates a direct role for the bicarbonate sensor in stimulating protein dephosphorylation. The rapid dephosphorylation is paralleled by previous reports that merocyanine reported boar sperm membrane fluidisation occurs within minutes after bicarbonate addition (Gadella and Harrison, 2000).

However, treatment of sperm with dbcAMP/IBMX in N conditions did not result in dephosphorylation demonstrating that elevation of cAMP alone was not sufficient to cause dephosphorylation. This further supports the observation that the bicarbonate sensor is a critical regulator of the changes that occur inside sperm cells undergoing capacitation. Interestingly, addition of dbcAMP/IBMX to the incubation media of boar sperm showed an increase of protein tyrosine phosphorylation of

multiple bands during capacitation and increased the phosphorylation in the posterior ring, midpiece and principal piece. In contrast, no increased tyrosine phosphorylation was reported in N sperm. This increase of tyrosine phosphorylation has been reported in boar sperm incubated with dbcAMP/IBMX (Harayama et al., 2004a).

One of the dephosphorylation events that were detected by the phospho ATM/ATR substrate antibody was independent of the phosphatase inhibitor calyculin A while the dephosphorylation of the other proteins was sensitive to this compound (Figure 4.10). This suggests that two different pathways leading to S/T protein dephosphorylation: one involving either PP1 and/or PP2A and one that is independent. Calyculin A has previously been used as a phosphatase inhibitor in boar sperm and greatly enhanced the phosphorylation of S/T PKA proteins (Harrison, 2004) and also caused increased sperm motility (Holt and Harrison, 2002). More recently, a study by Adachi *et al.* (2008) using immunolocalisation but not immunoblotting reported S/T protein dephosphorylation in the post-equatorial region during capacitation. These proteins showed increased phosphorylation when sperm were incubated with calyculin A. It is possible that one of the bands observed in our study may also be the protein(s) observed by (Adachi et al., 2008) and suggests a possible functional role for these phosphorylated proteins before and after ejaculation in boar sperm.

Although the role of protein phosphatases as components of signalling and regulatory pathways in other cell types is well known, relatively little is known about phosphatases in spermatogenesis and sperm. Inhibition of phosphatase activity resulted in initiation and stimulation of motility in bovine cells (Huang et al., 2005;

Vijayaraghavan et al., 1996), suggesting that phosphatases have an important role in regulation of sperm kinetic activity. Additionally, inhibition of S/T protein phosphatases has been found to induce capacitation associated signalling (Krapf et al., 2010). Protein phosphatases have been also localised in the sperm head which suggests a possible role in the AR or other head specific events (Adachi et al., 2008; Huang et al., 2002). Several pharmacological inhibitors of phosphatases have been used to investigate the mechanism of S/T phosphatases in regulating protein phosphorylation. These include calyculin A (Adachi et al., 2008; Carrera et al., 1996; Goto and Harayama, 2009; Harrison, 2004) and okadaic acid (Carrera et al., 1996; Jha and Shivaji, 2002b). Calyculin A is a cell permeable PP1/PP2A inhibitor whereas okadaic acid is protein phosphatase 2A. In this study, no changes in sperm motility were reported between sperm incubated under C conditions with and without calyculin A. This is in contrast with a study in the mouse when sperm incubated with calyculin was reported to become hyperactivated due to activated phosphorylation of flagellar proteins (Goto and Harayama, 2009).

In conclusion, S/T protein dephosphorylation in sperm incubated under C conditions has been reported for the first time in boar sperm. It affects multiple proteins and this observation has not previously been reported in mammalian sperm. Interestingly, this data showed two pathways of protein dephosphorylation with one regulated by PP1 and/or PP2A. Identification of these proteins may shed further light on the possible function of such dephosphorylation events during sperm capacitation or on the role of the (S/T) protein phosphorylation in N conditions prior to deposition in the female tract.

Having demonstrated dephosphorylation multiple proteins in boar sperm incubated under C conditions, the next step was to identify these proteins. Unfortunately the size of these proteins did not suggest any likely candidates based on the literature. Furthermore, while these are kinase substrate antibodies, it is possible that these proteins do not lie downstream of the kinases that denote the name of the antibody. This was shown to be true for the phospho (S/T) Akt substrate antibody which also recognises phosphorylation of S6 which is regulated by S6 kinase not Akt (Kane et al., 2002). Thus, the localisation of the dephosphorylated proteins in sperm fractions was the next investigation in **Chapter 5**. The importance of the dephosphorylated proteins localisation was to facilitate the identification of these proteins.

CHAPTER 5

Subcellular localisation of bicarbonate-dependent serine/threonine (S/T) dephosphorylated proteins

5.1 Introduction

The mammalian sperm is a highly differentiated cell whose structural features have been extensively investigated at the ultrastructural level. The role of most sperm compartments and membrane domains in the fertilisation process is well established (Yanagimachi, 1994). The localisation of proteins in a specific region of the cell may imply a possible function. Therefore sperm cells were fractionated in this chapter in order to gain information about the localisation and the possible function of the S/T dephosphorylated proteins detected in **Chapter 4.**

Many methods have been developed to separate sperm heads and tails such as sonication (Henle et al., 1938), pressure cavitation (Buhr et al., 1989), and the use of detergents or proteolytic enzymes (Gall et al., 1975). Several studies have separated sperm to heads and tails in order to extract and identify particular proteins localised in these fractions in murine (Baker et al., 2006), boar (Harrison, 2004; Lecuyer et al., 2000), human (Lecuyer et al., 2000) and bovine sperm cells (Hinsch et al., 2003). In addition, the apical plasma membrane of sperm head has been isolated using nitrogen cavitation to identify proteins associated with the plasma membrane and infer a role in zona binding or other sperm surface junctions (Buhr et al., 1989; Flesch and Gadella, 2000; Flesch et al., 1999; Flesch et al., 1998; Flesch et al., 2001a). The assessment of purity of sperm fractions is very important as the level of contamination that may occur during sperm subcellular fractionation should be known. Several marker enzymes have been widely used to measure the purity of sperm fractions especially of the plasma membrane following fractionation (Flesch et al., 1998; Millette et al., 1980; Thomas et al., 1997).

In **Chapter 4**, six (S/T) phosphorylated proteins were shown to be dephosphorylated following incubation in C conditions. These proteins had molecular weights of 105, 97, 96, 90, 64 and 55 kDa. The dephosphorylation of the phospho (S/T) proteins pp97, pp96, pp90, pp64 and pp55 were all found to be inhibited by calyculin A whereas pp105 was not affected by the same phosphatase inhibitor. This suggested that two pathways of S/T protein dephosphorylation with one regulated by PP1 and/or PP2A. This chapter will only focus on the five dephosphorylated proteins inhibited by calyculin A. The localisation of these proteins was mainly in the sperm heads including the apical plasma membrane and post-equatorial region as detected by indirect immunofluorescence. This may suggest a possible role involved in sperm-oocyte interaction. There is a clear issue with whole cell lysate studies as no definitive information about the subcellular localisation of the protein identified is provided. Despite immunofluorescence was used to detect the localisation of the dephosphorylated proteins, the differences between the fluorescence in N and C sperm cells was not always clear enough. Moreover, immunofluorescence alone was not enough to detect a particular protein in the sperm region. Therefore, to determine the possible function of these proteins in capacitation, subcellular fractionation is very valuable to isolate the sperm heads and tails and localise each of these proteins to the related fractions using immunoblotting.

The main purpose of this chapter was to separate sperm to heads and tails in order to localise them in sperm fractions. This localisation of the dephosphorylated proteins in both sperm head and tail fractions would facilitate the identification of these proteins by proteomic analysis and immunoblotting. The sperm head and tail

fractions were lysed and blotted with phospho PKA Ab to localise pp97, pp96, pp90, pp64 and pp55. This localisation was then used to inform the decision for the best protein candidates of these dephosphorylated proteins.

5.2 Aims

- Achieve subcellular fractionation of sperm cells and assess the purity of the fractions
- Localise the (S/T) pp97, pp96, pp90, pp64 and pp55 using subcellular fractions of heads and tails

5.3 Material and methods

5.3.1 Boar sperm fractionation

Fractionation of boar sperm was achieved by the development of a modified approach based on previous protocols (Baker et al., 2006; Harrison, 2004; Hinsch et al., 2003) and is summarised in Figure 5.1. Frozen sperm pellets (2 x 10^8) from incubations carried out under N conditions as described in **Section 2.2** were resuspended in 1.5 ml ice-cold PBS solution and sonicated (Sonopuls, Bandelin Electronic, Berlin, Germany, 1 min, 2 cycles and power 60 %). This was sufficient to detach all heads from tails as analysed using light microscopy. Subsequently, these fragmented materials were separated into head and tail fractions using differential centrifugation. The 1.5 ml sonicated sperm suspension was layered on the top of 5 ml 100% Percoll (prepared by diluting 12 parts Percoll with 1 part of 10X M **Section 2.1** and centrifuged at 2100 *g* for 45 min at 4 °C. The supernatant consisting of an enriched sperm flagellum fraction and was subjected to further centrifugation at 19, 285 *g* (which is the maximum speed in the centrifuge) for 40 min at 4 °C (Figure 5.1G and H). This procedure was performed twice to purify the tail fraction. The pellet, consisting of the head fraction was subjected to Percoll gradient centrifugation to purify the heads (Figure 5.1D and E). The purity of head and tail fractions was initially determined using light microscopy. Finally, purified sperm flagellum (Figure 5.1I) and heads (Figure 5.1F) were stored at -20 °C for subsequent use.

As previously mentioned, the purity of the heads and tails fractions was initially determined using light microscopy. Secondly, the purity of the heads and tail preparations was assayed by immunoblotting with an (acrosin) Acr-2 monoclonal Ab

or calicin Ab (CALI) which are both head proteins or with an α-tubulin Ab which is a tail protein.

5.3.2 1DE and immunoblotting

Isolated sperm head or tail pellets (1 x 10^8) were resuspended in 100 µl sample buffer (NuPAGE LDS, Invitrogen) and heated at 70 °C for 10 min. The sample was recentrifuged at 10,000 *g* for 5 min to remove non-solubilised material. Sperm proteins were separated by 1DE **Section 2.4** and then subjected to immunoblotting **Section 2.5**.

The primary antibodies were used at 1:1000 dilution [PKA sub-Ab, Acr-2 Ab (abcam, ab1900) and CALI Ab (abcam, ab75208)]. The secondary antibodies were diluted 1:10,000 (goat anti-rabbit for PKA substrate Ab and CALI Ab) or (goat anti-mouse for Acr-2).

5.3.3 Commassie blue staining

Proteins were visualised by commassie blue staining (Colloidal blue staining kit, Invitrogen). Gels were fixed with methanol, water and acetic acid for 10 min according to the manufacturer's instruction, then stained with buffer A for 10 min and then with buffer B for approximately 3 h before destaining with water.

5.3.4 Indirect immunofluorescence (IIF)

For more details about IIF see **Section 2.6**. All primary antibodies were used at 1:100 dilution and secondary antibodies were goat-anti-mouse Alexa Fluor 488-

conjugated antibody (Invitrogen; 1:300 dilution) for the CALI Ab, Acr-2 and α-tubulin Ab.

5.4 Results:

5.4.1 Whole sperm fractionated to heads and tails

Sonication of intact boar sperm (Figure 5.1A) resulted in fractions of heads and tails as revealed by light microscopy inspection (Figure 5.1B and 5.2A). The heads were separated from the tails at the posterior ring (PR) which remained connected with the tail (Figure 5.2E) and the heads were separated in the post-equatorial region (PE) (Figure 5.2D). Further centrifugation of the sperm resulted in tails in the interface layer and heads in the pellet (Figure 5.1C). A highly enriched head fraction approximately (90 % purity) was recovered in 100% Percoll gradient buffer at 2,100 *g* (Figure 5.1D). However, this fraction was still contaminated with some sperm tails. This mixture of tails and sperm heads was subjected to further Percoll gradient centrifugation. The sperm tail fraction formed a thin layer above the Percoll interface (Figure 5.1E). This sperm tail fraction was discarded and heads obtained from the pellet with a final pellet containing pure heads as determined by light microscopy (Figure 5.1F and 5.2B). On the other hand, the highly enriched sperm flagellum fraction approximately (90% purity) (Figure 5.1G) was centrifuged at 19.200 *g* for 45 min to further purify the sample (Figure 5.1H) and yielded a pure tail pellet as determined by light microscopy (Figure 5.1I and 5.2C)

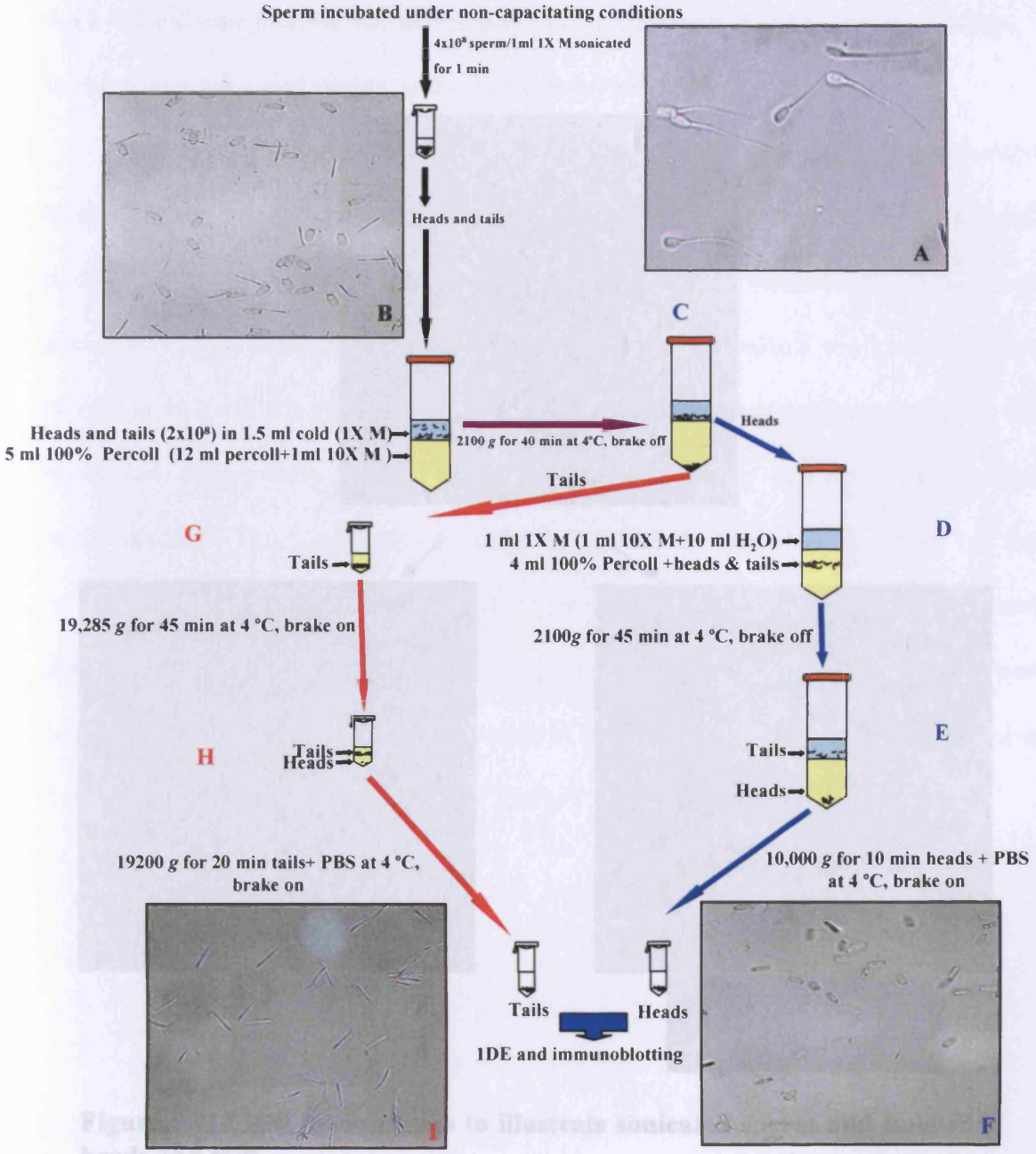

Figure 5.1: A schematic diagram to illustrate the subcellular fractionation of sperm into heads and tails.

(A) 4 x 10^8 sperm in1 m1X M sonicated for 1 min at 2 cycles 4 °C. **(B)** Following sonication (2 x 10^8) heads and tails in 1.5 ml cold 1X M media were layered on 5 ml 100 % Percoll at 2100 *g* for 40 min at 4 °C, brake off . **(C)** The pellet consisting of the head fraction and the interface consisting of an enriched sperm tail fraction. **(D)** The pellet consisted of the head fraction was subjected to Percoll gradient centrifugation to purify the heads completely from tails **(E)** and **(F).** **(G)** The tail fraction was subjected to further centrifugation at 19,285 *g* for 40 min at 4 °C **(H)** and **(I).** Blue coloured numbers are refer to heads and red numbers refer to tails.

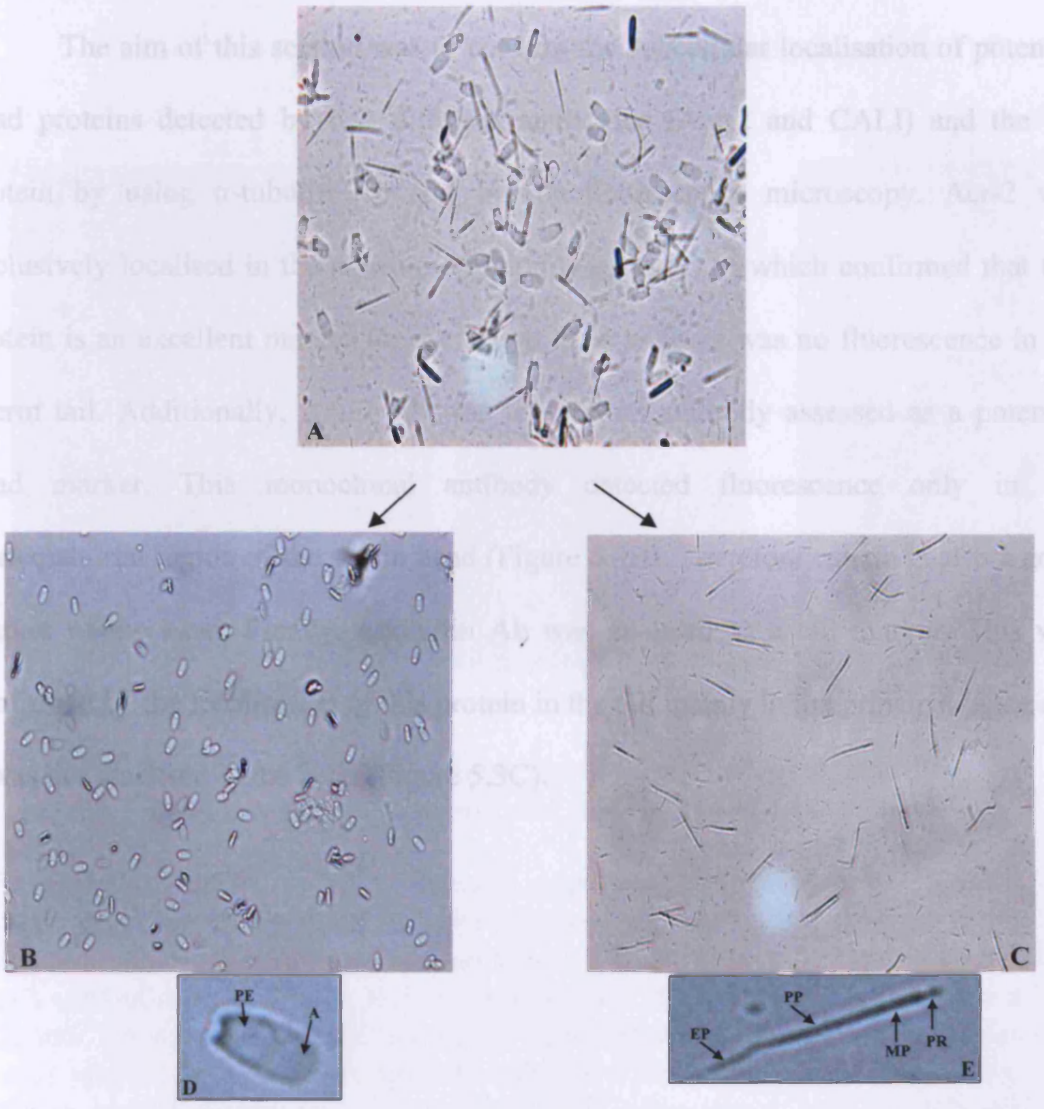

Figure 5.2: Light micrographs to illustrate sonicated sperm and isolated heads and tails.

Following sperm sonication of heads and tails **(A)** were separated by differential centrifugation. The pellet consisted of a pure head fraction with no tails **(B)** and the supernatant consisted of a pure sperm tail fraction with no heads **(C)**. **(D)** represents an individual head fraction and **(E)** represents an individual tail fraction. Key: A indicates, acrosome; PE, post-equatorial region; PR, posterior ring; MP, midpiece; PP principal piece and EP; endpiece.

5.4.2 Subcellular localisation of acrosin was in the sperm head acrosome, calicin in the post-equatorial region and α-tubulin in the tail

The aim of this section was to confirm the subcellular localisation of potential head proteins detected by two different antibodies (Acr-2 and CALI) and the tail protein by using α-tubulin Ab and immunofluorescence microscopy. Acr-2 was exclusively localised in the acrosome region (Figure 5.3A) which confirmed that this protein is an excellent marker for the sperm head as there was no fluorescence in the sperm tail. Additionally, CALI Ab was the second antibody assessed as a potential head marker. This monoclonal antibody detected fluorescence only in the postequatorial region of the sperm head (Figure 5.3B). Therefore calicin is also a good marker of the head. Finally, α-tubulin Ab was assessed as a tail marker. This was confirmed by the localisation of this protein in the tail mainly in the principal piece and it was not localised in the head (Figure 5.3C).

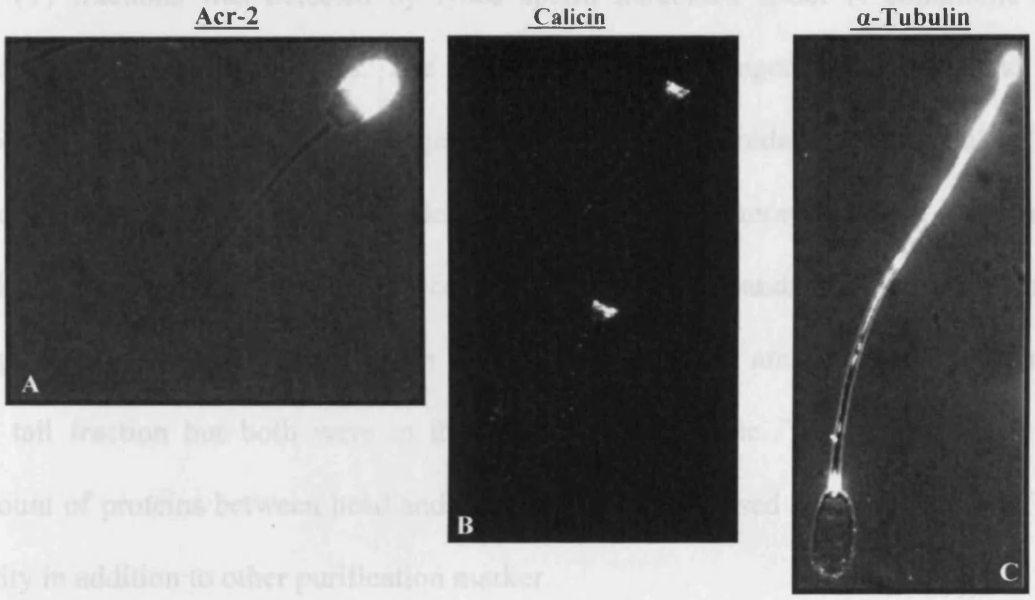

Figure 5.3: Differential subcellular localisation of proteins used as markers for the sperm head (acrosin and calicin) and tail (α-tubulin).

N sperm (1 x 10^5) were gently smeared onto a microscopic slide and allowed to air dry. Slides were fixed in 100 % methanol for 5 min and allowed to air dry for 1 h. Slides were then incubated for 1 h with **(A)** Acr-2, **(B)** calicin and **(C)** α-tubulin antibodies at 38.5 °C in a wet box. Slides were incubated for a further 1 h with goat-anti-mouse Alexa Fluor 488-conjugated antibody. Slides were washed with PBS and mounted with Slow Fade Light antifade solution. Slides were assessed by epifluorescence UV microscopy. **(A)** and **(C)** are paired images (fluorescence and light). **(B)** is **a** fluorescence-only image. The results shown were repeated of at least three experiments performed with different sperm samples

5.4.3 Head and tail fractions contained different proteins and fewer proteins then whole sperm

The amount and distribution of proteins in whole sperm (W) and head (H) and tail (T) fractions was detected by lysed sperm incubated under N conditions and heating at 70 °C for 10 min. The sample was recentrifuged for 5 min, and the supernatant was used for 1DE. The gel was stained with colloidal commassie blue. The amount of the protein bands in whole sperm (Figure 5.4) is more than that in the head and tail fraction. The head fraction contained less protein bands than the tail fraction (Figure 5.4). In addition, the protein bands in head fraction are different from that in the tail fraction but both were in the whole sperm sample. This difference in the amount of proteins between head and tail fractions can be used as an indication of the purity in addition to other purification marker.

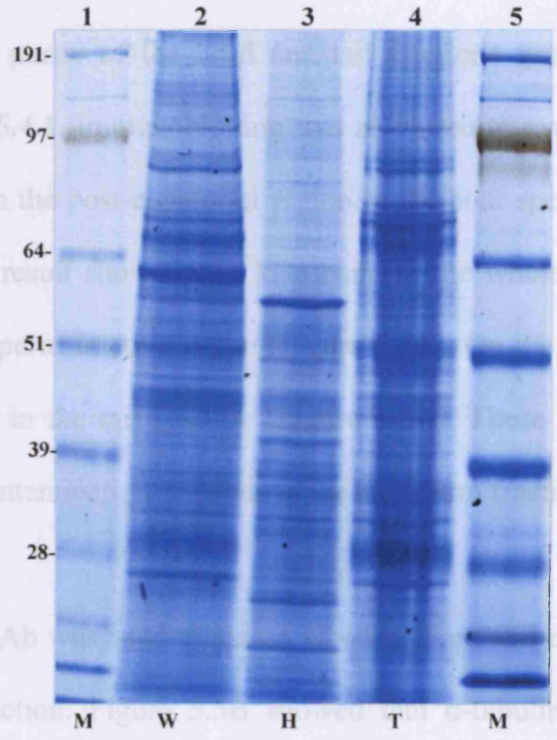

Figure 5.4: Analysis of proteins in whole sperm, heads and tails by 1DE and commassie blue-stained gel of the non-capacitated (N) boar sperm subcellular fractions.

Lane 1 and 5, SeeBlue marker **(M)**; lane 2, 2 x 10^6 whole sperm **(W)**, lane 3, 2 x 10^7 head **(H)** fraction, lane 4, 2 x 10^7 tail **(T)** fraction. Whole sperm and the fractions were subjected to 1DE as described in **Section 2.4**.

5.4.4 Calicin was only detected in whole sperm and the head fraction and α-tubulin was only detected in whole sperm and the tail fraction

Although the purity of the head and tail fractions was assessed under light microscopy **Section 5.4.1**, immunoblotting was also used to evaluate the purification. Calicin is localised in the post-equatorial region in the boar sperm head (Figure 5.3b) and immunoblotting result showed p55 kDa band in the whole sperm lysate (Figure 5.5A1) and in head sperm fraction lysate (Figure 5.5A). On the other hand, no calicin protein was detected in the tail fraction (Figure 5.5A). These results confirmed that there was no cross contamination of heads in the tail sperm fraction.

An α-tubulin Ab was used to assess whether there was cross-contamination of tails in the head fraction. Figure 5.5B showed that α-tubulin Ab was exclusively localised in the sperm tail which can be used as a marker to detect the purity of the head fraction. The head sperm fraction showed that there was no cross contamination with tail fraction (Figure 5.5B). In contrast, whole sperm (Fig 5.5B) and tail fraction (Fig 5.5B) showed a clear band of tubulin at a p50.

Figure 5.3A revealed that Acr-2 was localised in the acrosome region of the sperm head. This localisation also used as a marker of the head and as an evidence of tail purification. Figure 5.5C revealed Acr-2 proteins in whole, head and tail fractions. This monoclonal antibody was very sensitive and could detect a very small amount of Acr-2 proteins in the sperm fraction and the protein bands are very strong and could be developed within few seconds. This indicated that this antibody was not specific and reliable to be used as a head marker. This is consistent with the observation by other

workers using the same antibody to detect acrosin in boar sperm (Dr.Bart Gadella, Utrecht University, personal communication). Therefore, this antibody was discarded as a head marker and alternatively CALI Ab was used.

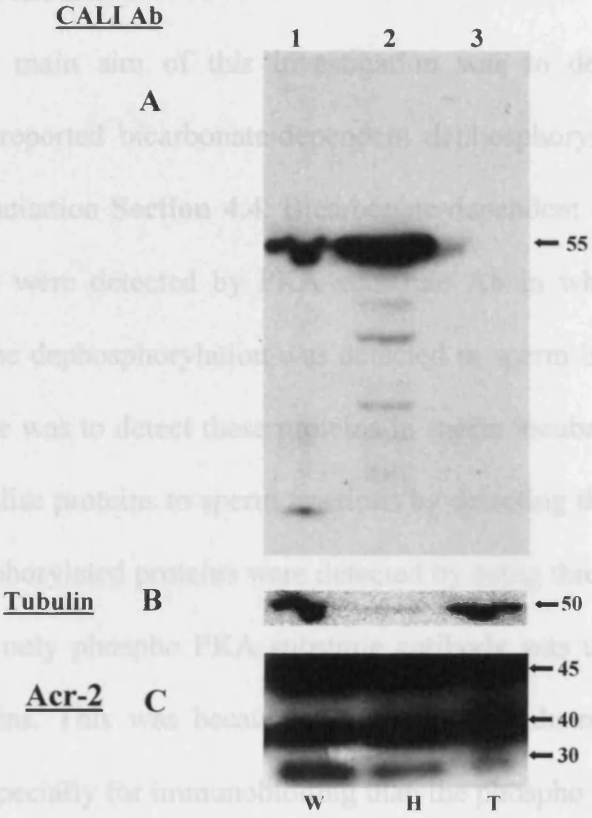

Figure 5.5: Subcellular localisation of potential head (calicin and acrosin) and tail (α-tubulin) markers in sperm.

lane 1, (2 x 10^6) whole sperm (W) lane 2, (2 x 10^7) head fraction (H) and lane 3, (2 x 10^7) tail fraction (T). Whole sperm and the fractions were subjected to 1DE as described in **Section 2.4**. The gels were transferred to membrane, blocked for 1 h and immunoblotted. **(A)** Immunoblotted with CALI Ab. **(B)** Stripped membrane immunoblotted with α-tubulin Ab. **(C)** Immunoblotted with Acr-2 Ab. The results shown were repeated at least 5 times with different sperm samples.

5.4.5 pp97, pp96 and pp64 are localised to the head whereas pp90 and pp55 are localised to the tail

The main aim of this investigation was to detect the localisation of the previously reported bicarbonate-dependent dephosphorylated proteins in boar sperm during capacitation **Section 4.4**. Bicarbonate-dependent dephosphorylated proteins in boar sperm were detected by PKA substrate Ab in whole, head and tail fractions. Although the dephosphorylation was detected in sperm incubated under C conditions, the aim here was to detect these proteins in sperm incubated under N conditions to be able to localise proteins to sperm fractions by detecting the phosphorylation. Although the dephosphorylated proteins were detected by using three different phospho substrate antibodies, only phospho PKA substrate antibody was used in this chapter to detect these proteins. This was because phospho PKA substrate antibody is good quality antibody especially for immunoblotting than the phospho Akt substrate antibodies.

The immunoblotting of whole sperm showed pp97, pp96, pp90, pp64 and pp55 compared with the localisation of pp97, pp96 and pp64 in the head fraction and pp90 and pp55 to the tail (Figure 5.6A).

Immunoblotting was also used to evaluate the purification of head and tail fractions. Immunoblotting with calicin Ab showed that p55 in the whole sperm and head sperm fraction lysate. On the other hand, calicin was detected in the tail fraction (Figure 5.6B). These results confirmed that there was no cross contamination of heads in the tail sperm fraction with heads. The α-tubulin Ab detected p50 in the whole

sperm and tail fractions but was not detected in the head sperm fractions. This showed that there was no cross contamination of the head fractions with tails (Figure 5.6C).

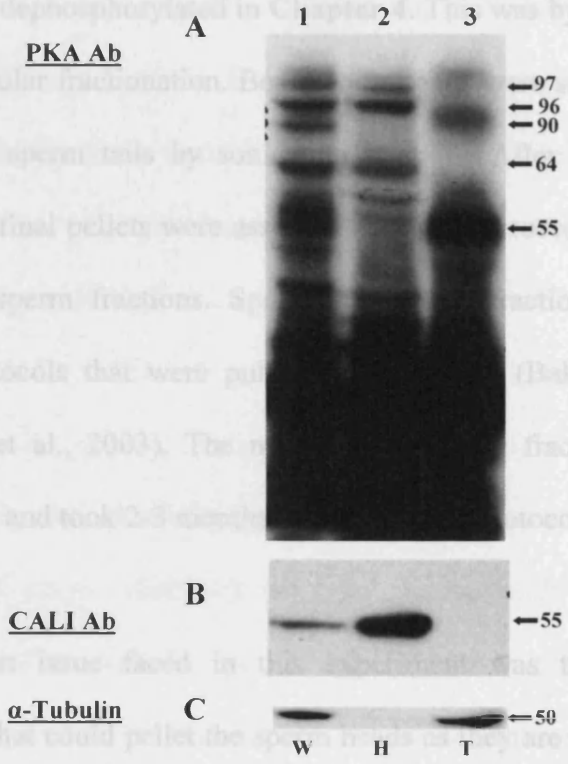

Figure 5.6: Subcellular localisation of pp97, pp96 and pp64 to the head and pp90 and pp55 in the tail detected by phospho (S/T) PKA substrate antibody.

lane 1, (2 x 10^6) whole sperm (W) ; lane 2, (2x10^7) head fraction (H) and lane 3, (2 x 10^7) tail fraction (T). Whole sperm and the fractions were lysed in sample buffer (NuPAGE LDS; Invitrogen) and heated at 70 °C for 10min. The sample was recenterfuged for 5min, and the supernatant was used for 1DE. The gels were transferred to membrane, blocked for 1 h and immunoblotted. **(A)** Immunoblotted with PKA Ab. **(B)** Immunoblotted with CALI Ab. **(C)** Stripped membrane immunoblotted with α-tubulin. The results shown were repeated at least 5 times with different sperm samples.

5.5 Discussion

This chapter sought to elucidate the localisation of pp97, pp96, pp90, pp64 and pp55 that were dephosphorylated in **Chapter 4**. This was by isolating sperm heads and tails by subcellular fractionation. Boar sperm heads were successfully and completely detached from sperm tails by sonication at 4 °C. After several centrifugation and washing steps, final pellets were assessed by light microscopy to initially evaluate the purity of the sperm fractions. Sperm subcellular fractionation was performed by modifying protocols that were published previously (Baker et al., 2006; Harrison, 2004; Hinsch et al., 2003). The modification of the fractionation method was not straightforward and took 2-3 months to establish the protocol.

The first issue faced in this experiment was to create Percoll gradient centrifugation that could pellet the sperm heads as they are heavier and the sperm tails moved above the solution because they are lighter than heads. To achieve this, the heads and tails were firstly layered on 75 % of Percoll then centrifuged. This dilution was not enough to pellet pure sperm heads. Therefore, the concentration was changed to 100% (12 ml Percoll + 1 ml 10X M). The next challenge in this experiment was the use of centrifuge force. Isolating pure sperm heads needed about 2,000 *g* to be achieved, whereas tail fractions were isolated at 19.285 *g* (this was the maximum force in the centrifuge used). However, these final steps were achieved after many attempts to isolate the purest head and tail fractions. All these steps were performed on ice and without adding phosphatase or protease inhibitors. This was to avoid affecting the proteins of interest (pp97, pp96, pp90, pp64 and pp55) and to keep the fractions in the same experimental conditions as used in **Chapter 4**.

Although sperm heads and tail fractions were isolated and monitored by microscopy to confirm the purification and protein content a further crude assessment of purity. Colloidal coomassie blue staining was performed to ensure that the protein content was different between fractions. It was very clear that the localisation of protein bands in the head were different than that in the tail fraction (Figure 5.4). Overall, the number of protein bands in the tail fraction was more than that in the head fraction. This may indicate that the head and tail fractions in the gel contained different proteins.

A further step to confirm the purity was immunoblotting to detect whether soluble material may contaminate the head fraction with tails or vice versa. It was important to confirm that the head and tail fractions are pure enough in order to localise the S/T dephosphorylated proteins and avoid a possible cross-contamination between fractions which may yield false results. To assess purity and possible cross-contamination between fractions, specific proteins were tested in each fraction. These proteins were detected by antibodies against head and tail proteins. CALI Ab was chosen to assess the purity of tail fraction whereas tubulin antibody was used to assess the purity of head fraction. Both antibodies were tested for the localisation of the proteins in heads and tails to confirm their specificity. This approach of using antibodies against heads and tails fractions has not been widely used in assessing the purity of subcellular fractionation. Noteworthy, most studies monitored the purity of the fractions by microscopy only (Harrison, 2004; Hinsch et al., 2003).

The localisation of the bicarbonate-dependent dephosphorylated proteins pp97, pp96, pp90, pp64 and pp55 in head and tail fractions was detected by using phospho (S/T) PKA substrate antibody. The pp97, pp96 and pp64 were localised to the head and the pp90 and pp55 proteins were in the tail. The importance of localising these dephosphorylated proteins in heads or tails was to facilitate their identification by proteomic approaches in **Chapter 6**. Only phospho (S/T) PKA substrate antibody was used to detect the localisation of the dephosphorylated proteins in sperm fractions because the subcellular fractionation of sperm cells was time consuming and the pure fractions obtained were not enough to perform immunoblotting using the other two phospho Akt substrate antibodies. However, in **Chapter 4,** similar immunofluorescence localisation of the phospho (S/T) PKA and Akt substrate proteins was reported. This localisation may suggest that these proteins are localised similarly in head and tail fractions too. This cannot be confirmed unless immunoblotting of head and tail fractions are immunoblotted with phospho (S/T) Akt substrate antibodies.

CHAPTER 6

Use of proteomic analysis to attempt to identify the bicarbonate-dependent serine/threonine (S/T) dephosphorylated proteins

6.1 Introduction

Protein identification and quantification has been facilitated in recent years by proteomics and mass spectrometry (MS). Proteomics is the study of protein products expressed by the genome (Brewis and Brennan, 2010). In mammalian sperm, proteomic approaches have been used to discover potential target proteins for the development of contraceptive vaccines (Naaby-Hansen et al., 1997; Shibahara et al., 2002). Other studies have used proteomics to characterise functionally defective sperm (Barratt, 2008; Conner et al., 2007; Lefievre et al., 2003; Pixton et al., 2004). This technology was also used to probe signal transduction pathways such as identifying tyrosine phosphorylated proteins during human (Ficarro et al., 2003) and boar (Jones et al., 2008) sperm capacitation. Moreover, proteomics has also been used for mapping and/or characterising proteins putatively involved in post-testicular sperm maturation (Aitken et al., 2007; Jones et al., 2008; Starita-Geribaldi et al., 2001; Syntin et al., 1996).

Strategies for proteomic identification of proteins from cells include gel-based and gel-free proteomics. For gel-based proteomics, an extract of proteins is separated using 1DE or 2DE. Following separation, proteins are detected (for example by silver staining or coomassie blue staining) and gel spots or bands corresponding to one or several distinct proteins are excised from the gel and digested with trypsin. The resulting peptides are subjected to MS and the results are compared with protein databases to enable definitive protein identification (Brewis, 1999; Brewis and Brennan, 2010). The approach (LC-MS) is performed by sample trypsin digestion to produce "peptide soup" and gel-free peptides are separated by LC before MS analysis.

It is also possible to use 1DE combined with liquid chromatography (LC) and followed by MS (GeLC-MS/MS workflow) to identify proteins. GeLC-MS is performed firstly by separating proteins in 1DE before subjecting individual protein bands or gel regions to digestion. Resulting peptides are then separated by LC before MS analysis. Finally, tandem MS data is used to search proteins databases to achieve a match and therefore protein identification based on amino acid sequences derived from multiple MS/MS spectra [reviewed by (Brewis and Brennan, 2010)].

The aim of this chapter was to try to identify pp97, pp96, pp90, pp64 and pp55 characterised in **Chapter 4** using phospho (S/T) PKA substrate and phospho (S/T) Akt substrate antibodies. These proteins were localised in head or tail fractions by subcellular fractionation described in **Chapter 5** (summarised in Table 6.1). GeLC-MS was chosen for proteomic analysis as 1DE was used to characterise the S/T dephosphorylated proteins by immunoblotting and the same type of gel were used to excise the spots corresponding to the molecular weights of the dephosphorylated proteins.

Sperm were incubated under N conditions, solubilised and subjected to 1DE. Protein bands corresponding to the dephosphorylated S/T proteins were excised using the SeeBlue marker as a guide for the molecular weights. Following trypsin digestion the resulting peptides were separated by LC and subjected to MS. Candidate proteins identified were selected and further analysed with specific antibodies.

6.2 Aims

- Use proteomic approaches to try to identify the S/T dephosphorylated boar sperm proteins varying for 105-50 kDa
- Investigate whether candidate proteins identification are the dephosphorylated proteins reported in **Chapter 4**

6.3 Materials and methods

6.3.1 One dimensional electrophoresis (1DE)

For more details see **Section 2.4**

6.3.2 Two dimensional electrophoresis (2DE)

Protein extract equal to 3 x 10^6 cells was prepared for 2DE using the 2D Clean-Up kit (GE Healthcare) to remove interfering contaminants and the final pellet was resuspended in 125 ml 1DE LDS sample buffer with 50 mM dithiothreitol (DTT), 1 % (w/v) bromophenol blue and 0.5 % (v/v) Isoelectric focussing buffer (IPG) pH 3–10 NL (GE Healthcare). This was added to a 7 cm IPG strip holder and overlaid with an IPG strip (7 cm, pH 3–10 NL) and Dry Strip cover fluid. Following strip rehydration (12 h at 20 °C) IEF of the samples was performed on an Ettan IPG Phor II IEF system (GE Healthcare) using the following program: 1 h at 500 V; 2 h at 1000 V (gradient); 1 h at 1000 V, 2 h at 8000 V (gradient); 8 h at 8000 V. The focused IPG strip was equilibrated for 15 min in equilibration buffer 1X (v/v) 1DE LDS sample buffer followed by 15 min in equilibration buffer containing 125 mM iodoacetamide. Equilibrated IPG strips were transferred to the IPG well of NuPAGE 4–12 % Bis-Tris Zoom gels (Invitrogen) and proteins were separated for 1 h at 200 V.

6.3.3 Silver staining

Proteins were visualised by silver staining using a commercially available kit (GE Healthcare, UK) with the standard protocol except that glutaraldehyde was omitted from the sensitising step and formaldehyde excluded from the silver solution but increased in the developing step. This protocol is modified from (Shevchenko et

al., 1996). Gels were initially fixed with 40 % (v/v) ethanol and 10 % (v/v) acetic acid for 30 min and then sensitised with 0.2 % (w/v) sodium thiosulphate, 30 % (v/v) ethanol, 0.83 M sodium acetate for 30 min. Following three washes in distilled water the gel was subjected to the silver reaction (0.25 % (w/v) silver nitrate for 20 min) and subsequently washed a further two times. The gels were developed (0.24 M sodium carbonate, 0.015 % (w/v) formaldehyde) for 1 min and then for 3 min with a fresh solution. Development was quenched with 50 mM Ethylenediaminetetracetic acid (EDTA) for 10 min and the gel was stored in 1 % (v/v) acetic acid. Gels were scanned using an image scanner (UMAX PowerLook 1120, GE Healthcare) and saved as 256 greyscale (600 dots per inch).

6.3.4 In-gel trypsin digestion

This process was performed by Mrs Swee Nixon at Central Biotechnology Services (CBS) Proteomics Facility at Cardiff University. Gel plugs (1.5 mm diameter) of interest were excised using a manual spot cutting pipette (Spot Picker One Touch Plus; Web scientific Ltd) and the gel spots placed into 96 well plates. Destaining (removal of coomassie) was performed prior to in-gel trypsin digestion (for 2DE only). Gel plugs were covered with 50 µl of 50 % (v/v) acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for 1 h until no stain remained. The spots were then washed with 25 µl with of 25 mM NH_4HCO_3 and the supernatant was removed. The spots were dehydrated with 50 µl of ACN for 15 min and supernatant removed. The pellet was then rehydrated in 50 µl of 25 mM NH_4HCO_3 for 10 min and the supernatant was removed. The pellet was dehydrated in 50 µl of ACN for 15 min and the supernatant was removed. 25 µl 10 mM DTT in 25 mM NH_4HCO_3 was added to

the spots and incubated at 56 °C for 1 h. After the supernatant was removed, 25 µl 55 mM iodoacetamide in 25 mM NH_4HCO_3 added and incubated for 45 min at room temperature. The spots were washed with 25 µl of 25 mM NH_4HCO_3 for 10 min. The spots were dehydrated in 50 µl ACN for 15 min, the supernatant was removed and the spots rehydrated in 50 µl 25 mM NH_4HCO_3 and then dehydrated in 50 µl ACN for 15 min.

The dehydrated gel plugs were manually digested using a slightly modified version of the (Shevchenko et al., 1996) method. Gel plugs were digested with modified trypsin (V5111 or V5113; Promega UK Ltd) which was prepared as described by the manufacturer (6.25 ng/µl in 25 mM NH_4HCO_3). The plugs were then covered with 25 mM NH_4HCO_3 to keep the gel wet during digestion and incubated at 37 °C for 4 h in the incubator with polymerase chain reaction (PCR) adhesive film. Samples were then sonicated in water bath for 10 min to release the peptides out of the gel. The digestion buffer was removed and the peptides were dried at 60-65 °C for 45 min. Gel plugs were covered with 20 µl ACN and covered with PCR adhesive film until dried. ACN containing peptides was removed and dried in an oven at 66-65 °C for 30 min.

The dried peptides were resuspended in 50 % (v/v) ACN in 5 ml of 0.1% (v/v) trifluoroacetic acid (TFA) for MS analysis and paired samples corresponding to 10 % of the material (0.5 µl) and 35 % of material (0.5 µl following some evaporation of solvent) were spotted onto a 384 well MS plate. The samples were allowed to dry and

overlaid with α-cyano-4-hydroxycinnamic acid (CHCA, Sigma; 0.5 µl prepared by mixing 5 mg matrix with 1 ml of 50 % (v/v) ACN in 0.1 % (v/v) TFA).

6.3.5 LC-MALDI

LC-MALDI was performed by Dr Sanjay Khanna at Central Biotechnology Services (CBS) Proteomics Facility at Cardiff University. Digested peptides (entire samples) were separated on a nano-LC system (UltiMate 3000, Dionex, Sunnyvale, USA) using a short one-dimensional reverse phase method. Peptides were separated using a C18 column (PepMap 75 mmid, 30 cm, 3 mm, 100 Å, Dionex) at a flow rate of 300 nl / min. The buffers used were: A = 2 % ACN in water with 0.05 % (v/v) TFA and B = 90% ACN in water with 0.01 % (v/v) TFA. Peptides were separated using a single-step gradient from 0 to 50 % solvent B for 40 min. A chromatogram was recorded at 214 nm. Fractionation of the peptides into 8 second spots on a LC-MALDI sample plate was performed with a Probot microfraction collector (Dionex) starting at 20 min. and spotting for a further 20 min. CHCA (Sigma) was used as MALDI matrix (2 mg / ml in 70 % (v/v) ACN in 0.1 % (v/v) TFA containing 10 fmol / mL Glu-Fib) which was continuously added to the column effluent via a µ-tee mixing piece at a flow rate of 1.4 ml / min.

6.3.6 Mass spectrometry for protein identification

MS was performed by Dr Ian Brewis and Dr Keith Hart, Central Biotechnology Services (CBS) Proteomics Facility at Cardiff University and I observed this on several occasions. We used a Matrix assisted laser desorption ionisation time of flight-time of flight (MALDI TOF/TOF) mass spectrometer (Applied Biosystems 4800

instrument) with a 200 Hz solidstate laser operating at a wavelength of 355 nm (Bienvenut et al., 2002; Gluckmann et al., 2007; Medzihradszky et al., 2000). MALDI mass spectra and subsequent tandem MS (MS/MS) spectra of the 6 (LC-derived) or 8 (2DE SAMPLES) most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolution of each other were excluded from the selection and the peaks were analysed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700–4000 Da; focus mass 2000). In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.661026 Torr) and default calibration.

All MS data analysis was performed by myself following extended training. MS/MS queries were performed using the MASCOT database search engine v2.1 (Matrix Science, London, UK)(Perkins et al., 1999) embedded into Global Proteome Server (GPS) Explorer software v3.6 (Applied Biosystems) on the Swiss-Prot database. Searches were restricted to the mammalian taxonomy with trypsin specificity (one missed cleavage allowed), the tolerances set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/MS. Cysteine modification by iodoacetamide was employed as a fixed modification with methionine oxidation as a variable modification. Search results were evaluated by manual inspection and conclusive identification confirmed if there was high quality tandem MS (good *y*-ion) data for 2 or more peptides and a high peptide score C.I % usually above 99% calculated in GPS Explorer software, which is based on the MASCOT score.

6.3.7 Immunoblotting

For immunoblotting see **Section 2.5** and membranes were probed overnight with antibodies (Table 2.1). Membranes probed with secondary antibodies at 1:10,000 dilution for 1 h.

6.3.8 Indirect immunofluorescence

Incubated sperm cells (1 x 10^5) were gently smeared onto a microscopic slide (**section 2.6**) and incubated for 1h with different antibodies (Table 2.1) at 38.5 °C. Following incubation the slides were incubated for a further 1 h with secondary antibodies anti-mouse Alexa Fluor 488-conjugated antibody (Invitrogen; 1:300 dilution) for the HSP70/72, HSPA1L and with anti-goat Alexa Fluor 488-conjugated Ab (Invitrogen, diluted 1:300) for AKAP3 and AKAP4 antibodies.

6.3.9 Immunoprecipitation

Sperm pellets (1 x 10^8) incubated under N and C conditions **Section 2.2** were resuspended in 200 µl sample buffer (NuPAGE LDS, Invitrogen) and heated at 70 °C for 10 min. The sample was recentrifuged at 10,000 *g* for 5 min to remove non-solubilised material as a pellet. Sperm proteins in the supernatant equivalent to 1 x 10^8 cells were resuspended in 900 µl Tris buffer pH (6.8) with phosphatase inhibitors (cocktail 2 and 1 P5726) and protease inhibitor 10 µl/1ml (Sigma Aldrich, UK). 40 µl of protein G agarose beads (Sigma Aldrich, UK) were rotated for 2 h at 4 °C with the following controls included: 5 µl phospho (S/T) PKA substrate antibody and 200 µl (2 x 10^7 sperm) of N or C lysate. In addition, 40 µl of agarose beads were added to 200 µl of N or C lysate (2 x 10^7 sperm) and 5 µl phospho (S/T) PKA substrate antibody to

precipitate the pp97, pp96, pp90, pp64 and pp55. After 2 h rotation at 4 °C, beads were washed 3 times with Tris (pH 6.8) at 3,542 *g* (Beckman Coulter Allergra centrifuge) for 1 min. Agarose beads were resuspended in 100 µl 1DE LDS sample buffer and heated at 70 °C for 10 min. Sperm proteins in the supernatant equivalent to 3 x 10^6 cells were separated by 1DE **Chapter 2**. Moreover, N or C lysate with and without phosphatase and protease inhibitors equal to 3 x 10^6 cells were loaded as controls.

6.4 Results:

6.4.1 GeLC-MS identification of dephosphorylated protein candidates

1DE was performed as described in **Section 2.4** using boar sperm incubated under N conditions. N and C sperm protein lysates equal to 2 x 10^6 cells were loaded in parallel (three lanes each) to use them for silver staining and to pick the plugs for protein identification by mass spectrometry. The gel was stained with silver to visualise the proteins. The other 1DE was used to cut the plugs corresponding to molecular weights from 191 to 51 kDa (Figure 6.1). Not all spots were used but instead twelve plugs corresponding to the pp96, pp90, pp64 and pp55 detected by phospho (S/T) Akt substrate Ab and phospho (S/T) PKA substrate were selected. The peptides were separated by LC and subjected to MS/MS analysis (Figure 6.2). The GeLC-MS was repeated three times using (N) whole sperm cells.

This approach identified 37 proteins in total (Table 6.2). The next step after identifying these proteins was to choose the most likely candidates for the dephosphorylated proteins described in **Chapter 4**. This study could not investigate all the 37 candidates due to the limited time and funds available. Therefore it was important to limit the protein identifications to the molecular weights of the dephosphorylated proteins pp97, pp96, pp90, pp64 and pp55. Serum albumin and trypsin were excluded from the candidate list as these proteins were used in the incubation media or for sperm protein digestion, respectively. Finally, only proteins with two or more peptides with good quality data were chosen as possible candidates. The protein candidates selected were A-kinase-anchor protein 4 (AKAP4), A-kinase-anchor protein 3 (AKAP3), Heat shock protein 70 kDa protein 1 like (HS71L) and

Calicin (CALI) (Table 6.3). These candidates were then searched in the database for the detection of phosphorylation sites at S/T residues. This was performed by using a website (http://www.hprd.org/PhosphoMotif_finder) called PhosphoMotif Finder and contains known kinase/phosphatase substrates as well as binding motifs that are curated from the published literature. It reports the presence of any literature-derived motif in the query sequence. AKAP3, AKA4, HSP71L and CALI proteins were found to have phospho S/T sites at PKA substrate motifs (Table 6.4).

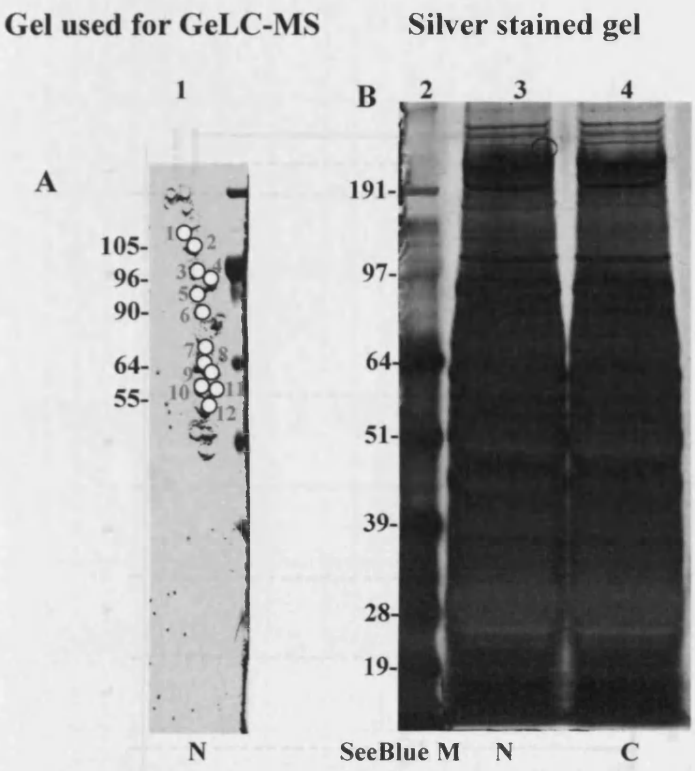

Figure 6.1: GeLC-MS protein identification of S/T dephosphorylated sperm proteins.

Non-capacitating (N) 2 x 10^6 and capacitating (C) 2 x 10^6 sperm solubilised with 1DE LDS sample buffer in parallel. The non-stained gel was used to cut the gel plugs corresponding to the bands of the S/T dephosphorylated proteins pp105, pp97, pp96, pp90, pp64 and pp55. The gel plugs (red coloured) was trypsin digested and identified by GelC-MS (A). The silver stained gel were used to confirm the presence of proteins (B). The results shown were repeated three times with different sperm samples.

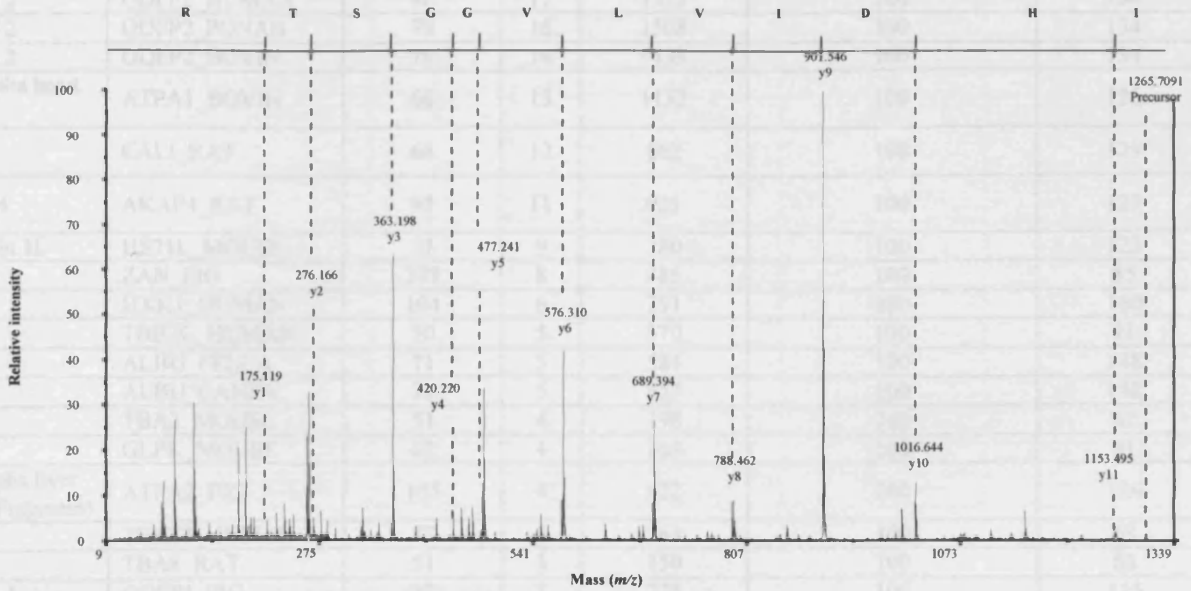

Figure 6.2: Example of tandem mass spectra (MS/MS) derived from one spotpeptide sequence of serine/threonine (S/T) dephosphorylated proteins in boar sperm using GeLC-MS.

MS/MS spectra of 1265 Da (**IHDIVLVGGSTR**) peptide of HSP71L-PIG from spot excised in corresponding to 64 kDa approximately.

	Protein name	Accession number	Molecular weight	Pep cnt	Total ion score	Total ion score confidence interval C.I%)	Best ion score	Best ion score C.I%	Spot number
1	Serum albumin	ALBU_BOVIN	71	25	2121	100	148	100	2 and 8
2	Outer dense fiber protein 2	ODFP2_RAT	96	17	1485	100	127	100	6
3	Outer dense fiber protein 2	ODFP2_MACFA	74	17	1514	100	134	100	6
4	Outer dense fiber protein 2	ODFP2_HUMAN	96	17	1523	100	134	100	6
5	Outer dense fiber protein 2	ODFP2_PONAB	79	16	1508	100	134	100	6
6	Outer dense fiber protein 2	ODFP2_BOVIN	76	16	1433	100	134	100	6
7	ATP synthase subunit alpha heart isoform, mitochondrial	ATPA1_BOVIN	60	13	1132	100	126	100	12
8	Calicin	CALI_RAT	68	12	862	100	129	100	10
9	A-kinase anchor protein 4	AKAP4_RAT	95	11	921	100	127	100	5, 6, 9 and 10
10	Heat shock 70 kDa protein 1L	HS71L_MOUSE	71	9	780	100	123	100	8 and 9
11	Zonadhesin	ZAN_PIG	279	8	485	100	85	100	7
12	Hexokinase-1	HXK1_HUMAN	104	6	391	100	160	100	5
13	Tubulin beta-2C chain	TBB2C_HUMAN	50	5	370	100	91	100	12
14	Serum albumin	ALBU_FELCA	71	5	584	100	148	100	8 and 9
15	Serum albumin	ALBU_CANFA	71	5	457	100	148	100	9
16	Tubulin alpha-3 chain	TBA3_MOUSE	51	4	198	100	62	100	12
17	Glycerol kinase	GLPK_MOUSE	62	4	263	100	91	100	12
18	ATP synthase subunit alpha liver isoform, mitochondrial (Fragment)	ATPA2_PIG	155	4	422	100	126	100	12
19	Tektin-3	TEKT3_HUMAN	57	3	163	100	63	99.9	12
20	Tubulin alpha-8 chain	TBA8_RAT	51	3	150	100	63	99.9	12
21	Outer dense fiber protein 1	ODFP1_PIG	32	3	225	100	115	100	11 and 12
22	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	G3PT_MACFA	45	3	200	100	99	100	12
23	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	G3PT_BOVIN	44	3	190	100	99	100	12
24	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	DHSA_MACFA	74	3	180	100	72	99.9	9
25	A-kinase anchor protein 4	AKAP4_HUMAN	96	3	240	100	127	100	6

	Protein name	Accession number	Molecular weight	Pep cnt	Total ion score	Total ion score confidence interval (C.I%)	Best ion score	Best ion score C.I%	Spot number
26	A-kinase anchor protein 3	AKAP3_MOUSE	97	3	218	100	84	99.9	5
27	Trypsin	TRYP_PIG	25	2	124	100	75	99.9	2
28	Tektin-3	TEKT3_MOUSE	57	2	107	100	63	99.9	12
29	L-amino-acid oxidase	OXLA_HUMAN	63	2	173.3	100	108	100	11
30	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	NDUS1_GORGO	80	2	147	100	92	100	6
31	60 kDa heat shock protein, mitochondrial	CH60-HUMAN	61	2	104	100	66	99.9	10
32	Serum albumin	ALBU_MOUSE	71	2	208	100	148	100	8
33	Angiotensin-converting enzyme, somatic isoform	ACE_RAT	151	2	86	100	51.3	99.8	4
34	Succinate dehydrogenase	DHSA_PIG	74	4	210	100	72	99.99	8 and 9
35	Tubulin alpha-3 chain	TBA3_MESAU	22	4	198	100	63	99.99	11 and 12
36	Amine oxidase	AOFA_PIG	60	2	120	100	86	100	11
37	Proteindpy-19 homolog 2	D19L2_HUMAN	88	2	114	100	79	99.99	12

Table 6.2: A list of the 37 proteins identified by GeLC-MS proteomic analysis.

Proteins (identified by immunoblotting in **Chapter 4**) were matched to a parallel silver stained 1DE gel and the relevant gel plug excised, digested and analysed by tandem mass spectrometry (Figure 6.1). Table 6.2 illustrates the 37 identified in this chapter. Pep ctn = number of different peptides identified from the protein.

Protein name	Accession number		Peptide sequence	Ion score	Expect value	Predicted molecular weight	Mass of Band excised
A-Kinase anchor protein 4	AKAP4_RAT	1	QNAADIMEAMLK	86	3.70e-07	96 kDa	pp97, pp96 and pp90
		2	EIVSDLIDSCMK	83	6.90e-08		
		3	QLDEAVGNMAR	73	8.10e-06		
		4	LVSALLGEK	69	2.60e-05		
		5	QLDEAVGNMAR	65	8.1e-06		
		6	LSSLVIQMAR	65	0.00049		
		7	TEGSVCLFK	50	0.0015		
Heat shock 70 kDa protein 1L	HS71L_PIG	1	IHDIVLVGGSTR	115	7.3e-11	71	pp64
		2	TTPSYVAFTDTER	113	7.5e-10		
		3*	IHDIVLVGGSTR	123	7.3e-11		
		4	IINEPTAAAIAYGLDK	100	1.3e-08		
		5	LVSHFVEEFKR	93	7.5e-08		
		6	LVSHFVEEFK	69	1.9e-05		
		7	LLQDYFNGR	61	0.00014		
		8	ATAGDTHLGGEDFDNR	55	0.002		
A-Kinase anchor protein 4	AKAP3_MOUSE	1	SVGEVLQSVLR	84	6e-07	97 kDa	pp97, pp96 and pp90
		2	EVVSDLIDSFMK	69	1.8e-05		
		3	QLDEAVGNVTR	65	4.7e-05		
Heat shock protein 70	HSP72_MOUSE	1	TTPSYVAFTDTER	113	7.5e-010	71 kDa	pp64
		2	IINEPTAAAIAYGLDK	100	1.3e-008		
Calicin	CALI_MOUSE	1	VVISEQNVEELLR	129	1.7e-011	68 kDa	pp55
		2	KGALLDSVVILGGQK	121	1e-010		
		3	VFVCGGVTTASDVQTK	97	2.4e-008		
		4	NVLAAVSPLVK	74	5.9e-006		
		5	EVSDLAYSGIR	71	1.3e-005		
		6	IHCNDFLIK	58	0.0003		
		7	CppVIFGR	58	0.00035		
		8	DYTINPNAYLLDQK	54	0.00052		
		9	NLYIVTGR	47	0.0019		
		10	GALLDSVVILGGQK	54	0.00055		
		11	NLYIVTGR	50	0.0019		

Table 6.3: Peptides have possible candidates of the pp97, pp96, pp90, pp64 and pp55 identified by GeLC-MS analysis.

Proteins (identified by immunoblotting in **Chapter 4** were matched to a parallel silver stained 1DE gel and the relevant gel plug excised from a non-stained gel, digested and analysed by tandem mass spectrometry (Figure 6.1). Table 6.3 illustrates the most likely candidates among the 37 proteins identified of the pp97, pp96, pp90, pp64 and pp55 detected by phospho PKA substrate antibody. 3* was used to show an example of peptide sequencing in Figure 6.2.

Accession number	Position in querey protein	Sequence in query protein	Corresponding motif described in the literature (phosphorylated residues in red)
CALI-HUMAN	146-149	KEVS	KXX[pS/PT]
	264-267	RPCS	RXXpS
	499-502	KTTT	KXX[pS/PT]
	542-545	KDYT	KXX[pS/PT]
	559-562	KWKT	KXX[pS/PT]
AKAP3-PIG	40-43	RVLS	KXX[pS/PT]
	170-173	KSLS	KXX[pS/PT]
	174-177	KVAS	KXX[pS/PT]
	214-217	KYKS	[R/K]X[pS/PT]
	222-225	KEST	KXX[pS/PT]
	234-237	RPAS	RXX[pS/PT]
	234-237	KLKS	KXX[pS/PT]
	310-313	KDTT	KXX[pS/PT]
	381-384	KLYT	KXX[pS/PT]
	399-402	KDKS	KXX[pS/PT]
	435-438	KICS	KXX[pS/PT]
	706-709	RPVS	RXXpS
	743-746	RGMS	RXXpS
	814-817	KGRS	RXX[pS/pT]
AKAP4-PIG	170-173	KSLS	[R/K]X[pS/PT]
	174-177	KVAS	KXX[pS/PT]
	214-217	KYKS	KXX[pS/PT]
	222-225	KEST	KXX[pS/PT]
	234-237	RPAS	RXXpS
	310-313	KDTT	[R/K]X[pS/PT]
	381-384	KLYT	KXX[pS/PT]
	399-402	KDKS	KXX[pS/PT]
	435-438	KICS	[R/K]X[pS/PT]
	442-447	KPET	KXX[pS/PT]
	706-709	RPVS	RXXpS
	743-746	RGMS	RXXpS
	814-817	KGRS	[R/K]X[pS/PT]
HS71L-PIG	238-241	RLVS	RXXpS
	274-279	RTLS	RXX[pS/PT]
	417-420	KRNS	KXX[pS/PT]
	417-420	KRNS	[R/K]X[pS/PT]

Table 6.4: A list of protein candidates CALI, AKAP3, AKAP4 and HS71L S/T phosphorylation sites in PKA motifs generated from the database.

6.4.2 AKAP4 proteins p96, p64 and p55 were detected by immunoblotting and were localised to the principal piece of sperm tails

In **Section 6.4.1**, the N sperm lysate was analysed by MASCOT searching and showed that AKAP4 was a possible candidate for pp97, pp96 and pp90 identified by the phospho PKA substrate Ab. Immunoblotting was performed using the N and C

sperm lysates and showed that proteins with p96, p64 and p55 were detected by AKAP4 Ab (Santa Cruz, Cat. #66308) (Huang et al., 2005) (Figure 6.3A).

Moreover, immunolocalisation of AKAP4 proteins using AKAP4 Ab was performed and detected fluorescence in the principal piece of the sperm tails (Figure 6.3B). This was in contrast to the localisation of pp97 and pp96 in the head fraction as revealed in **Section 5.5A** using phospho (S/T) PKA substrate Ab. Therefore, AKAP4 was excluded as one of the candidates for pp97 and pp96.

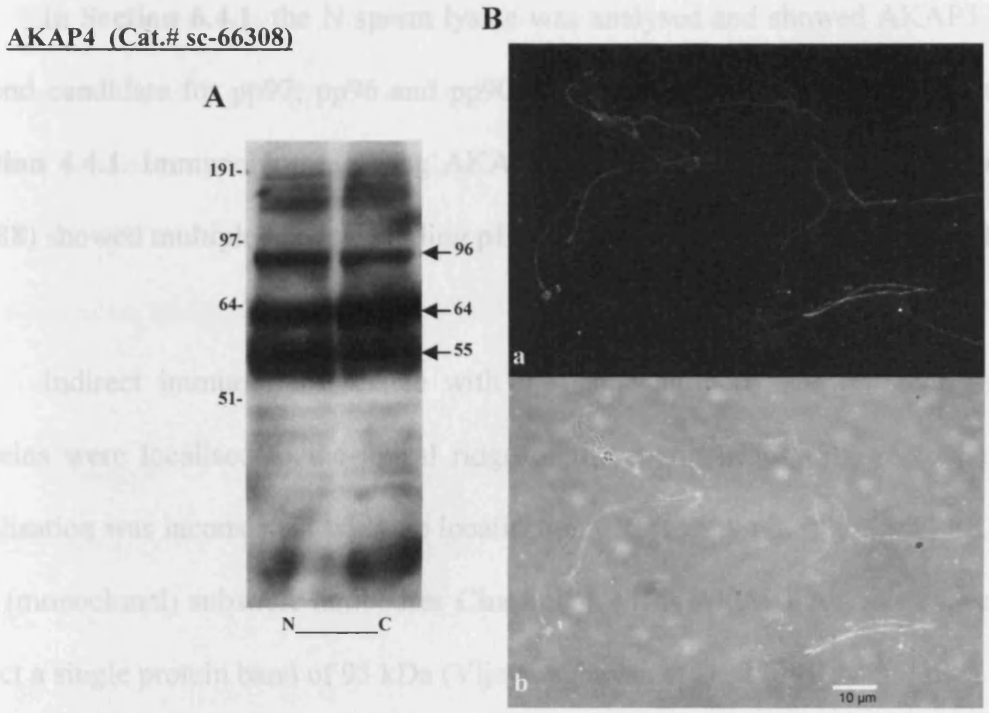

Figure 6.3: AKAP4 proteins p96, p64 and p55 were detected by immunoblotting and were localised to the principal piece of sperm tails.

Non-capacitating (N) sperm 1 x 10^6 and capacitating (C) sperm 1 x 10^6 sperm were solubilised and supernatant for 1DE. **(A)** Gel was transferred to a membrane, blocked and probed with AKAP4 Ab.**(B)** Immunolocalisation of AKAP4. **Ba** is fluorescence only image and paired image (fluorescence and light) is shown in **Bb** respectively. The results shown are representative of at least three experiments performed with different sperm samples.

6.4.3 Immunoblotting of AKAP3 using two different AKAP3 antibodies detected non-specific proteins and were localised to different sperm regions

In **Section 6.4.1**, the N sperm lysate was analysed and showed AKAP3 was a second candidate for pp97, pp96 and pp90 identified by phospho PKA substrate Ab **Section 4.4.1**. Immunoblotting using AKAP3 Ab (Santa Cruz Biotechnology, Cat. # 47788) showed multiple bands including p190, p96, p90, p85 and p45 (Figure 6.4A).

Indirect immunofluorescence with the same antibody showed that AKAP3 proteins were localised to the apical ridge of the sperm heads (Figure 6.4B). This localisation was inconsistent with the localisation detected by phospho (S/T) PKA and Akt (monoclonal) substrate antibodies **Chapter 4**. [This AKAP3 Ab was expected to detect a single protein band of 95 kDa (Vijayaraghavan et al., 1999)].

Because the AKAP3 Ab (Cat. #sc-47788) was unable to detect a protein of the correct size by immunoblotting, AKAP3 Ab (Cat. #Abcam19046) was used as an alternative antibody to detect the AKAP3 proteins in sperm. Immunoblotting of N and C sperm lysate showed p96, p64 and p55 bands (Figure 6.5A).

Immunolocalisation of the AKAP3 proteins using AKAP3 Ab (Cat. #Abcam19046) detected fluorescence in the principal piece of the sperm tails (Figure 6.5B). This was similar to the AKAP4 proteins detected with AKAP4 Ab (Figure 6.3A and B). This similarity was due to same peptides being shared between AKAP3 and AKAP4 proteins which are probably related to the same protein family. The AKAP3 antibody was raised against synthetic peptide **TPLQLLDWLMVNL**. This peptide corresponds to the C-terminal amino acids 841-853 of human AKAP3 (ExPASY)

whereas AKAP4 Ab was raised against a peptide mapping near the C-terminal of AKAP4 of human origin with no more information about the peptide sequence. Therefore, when the peptide sequence of the AKAP3 corresponding to the C-terminal amino acids 841-853 of human was compared with the AKAP4 corresponds to the C-terminal amino acids 841-853 of pig from UniProtKB/Swiss-Prot, the AKAP4 peptide TPLQ**LLDWLMVNL** was found to be shared with AKAP3 peptide (red letters) in 845-853 amino acides TRLQ**LLDWLMVNL** (Table 6.5).

Although the two AKAP3 antibodies used were potentially able to detect the AKAP3 proteins by immunofluorescence, immunoblotting results were non-specific overall. Therefore, AKAP3 is still a possible candidate for pp97, pp96 and pp90 but the lack of a good quality specific AKAP3 antibody prevented further investigation.

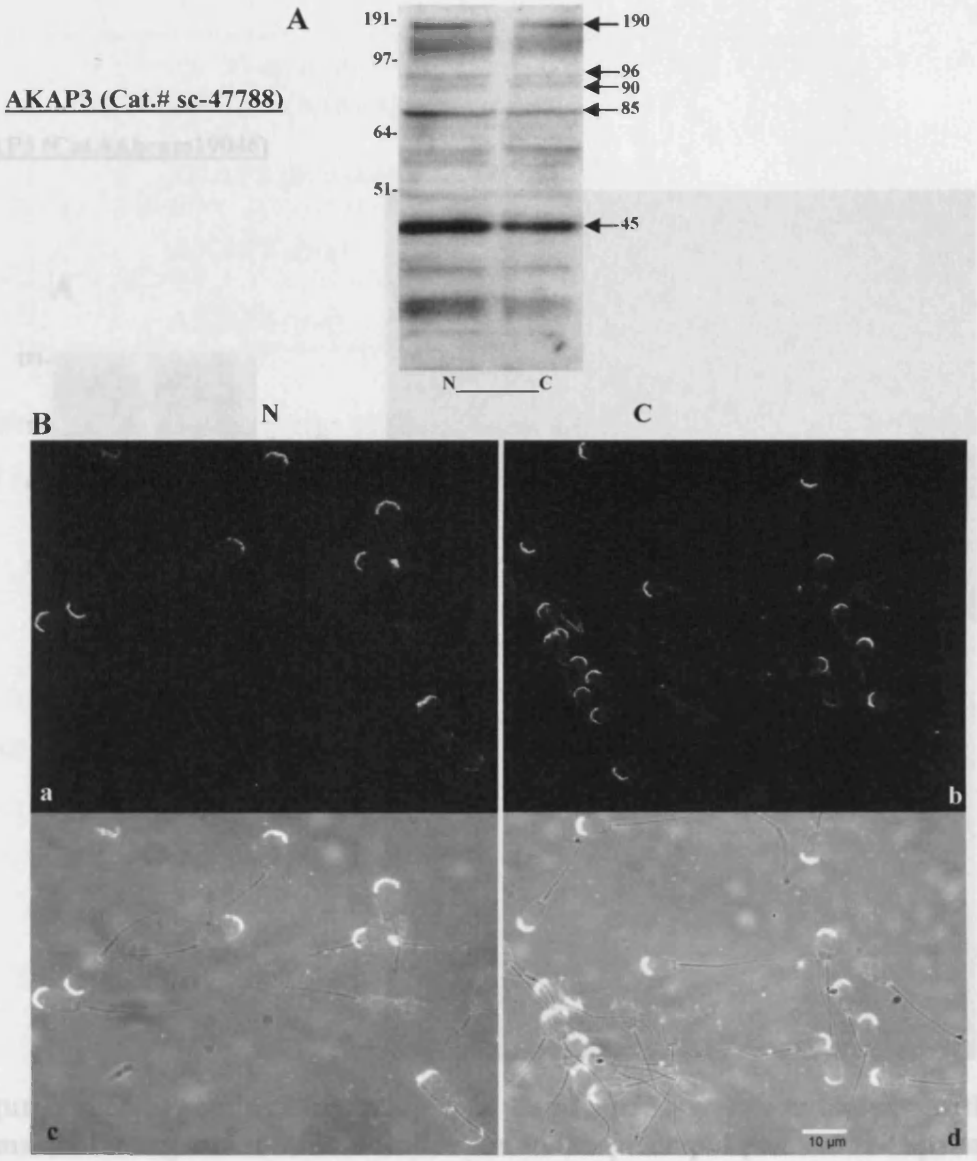

Figure 6.4: AKAP3 proteins were localised to the apical ridge of the sperm heads by using AKAP3 antibody but major proteins p190, p96, p90, p85 and p45 proteins were detected by immunoblotting.

(A) Non-capacitating (N) sperm 1 x 10^6 and capacitating (C) sperm solubilised and the supernatant was used for 1DE. Gel was transferred to membrane, blocked and probed with AKAP3 Ab (Santa Cruz biotechnology). **(B)** Immunolocalisation of AKAP3 proteins. **Ba** and **Bb** are fluorescence only image and paired images (fluorescence and light) are shown in **Bc** and **Bd** respectively. The results shown are representative of at least three experiments performed with different sperm samples.

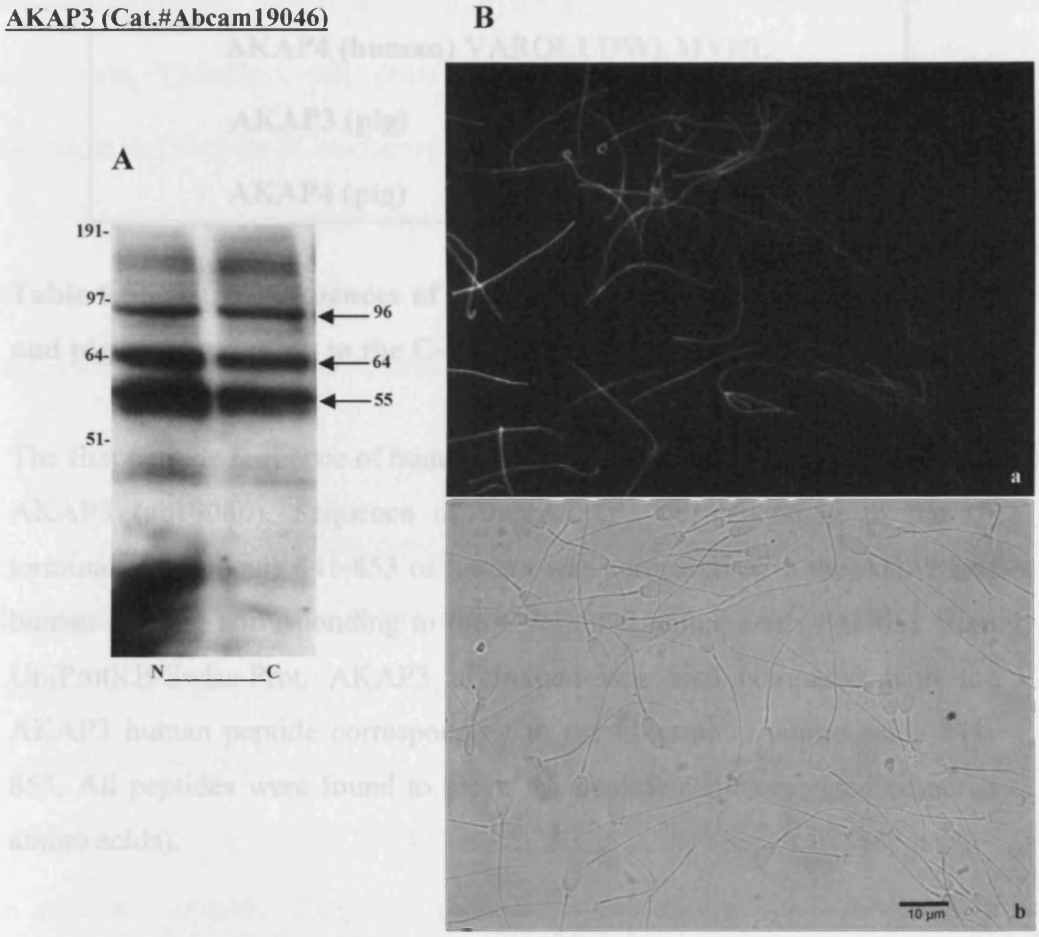

Figure 6.5: Proteins of p96, p64 and p55 were detected with immunoblotting and immunolocalisation to the principal piece of the sperm tails using AKAP3-abcam antibody.

(A) Non-capacitating (N) sperm 1 x 10^6 and capacitating (C) sperm 1 x 10^6 solubilised and the supernatant was used for 1DE. Gel was transferred to membrane, blocked and probed with AKAP3-abcam Ab. **B** Immunolocalisation of AKAP3-abcam Ab. **Ba** is a fluorescence only image and a paired image (fluorescence and light) is shown in a **Bb**. The results shown are representative of at least three experiments performed with different sperm samples.

C-terminal	841	845	853
AKAP3 (human)	TPLQLLDWLMVNL		
AKAP4 (human)	VARQLLDWLMVNL		
AKAP3 (pig)	TRLQLLDWLMVNL		
AKAP4 (pig)	MARKLLDWLMVNL		

Table 6.5: Shared sequences of AKAP3 and AKAP4 peptides of human and pig corresponding to the C-terminal amino acids 845-853.

The first peptide sequence of human was used to make the anti-goat antibody AKAP3 (ab19046). Sequence of the AKAP3 corresponding to the C-terminal amino acids 841-853 of human was compared with the AKAP4 of human and pig corresponding to the C-terminal amino acids 841-853 from UniProtKB/Swiss-Prot. AKAP3 of human was also compared with the AKAP3 human peptide corresponding to the C-terminal amino acids 841-853. All peptides were found to share the peptide sequences (red coloured amino acids).

6.4.4 Immunoblotting using calicin Ab detected p55 and was localised to the post-equatorial region of sperm heads

Calicin was a protein candidate for pp55 identified by GeLC-MS (**Table 6.2**). In mammalian sperm, calicin is a basic cytoskeletal protein which is localised only in sperm heads. Therefore, this protein was used as a head marker to assess the purification in **Chapter 5**. Immunoblotting using N whole sperm (W), head (H) and tail (T) protein extracts were separated by 1DE, transferred to a membrane and immunoprobed with calicin Ab (Abcam, Cat. #ab75208). A single p55 band was detected in the whole sperm and head but not in the tail (Figure 6.6A).

Indirect immunofluorescence of N sperm with calicin Ab was localised to the post-equatorial region of the sperm head (Figure 6.6B). Whilst this localisation was consistent with the fluorescence detected by phospho (S/T) Akt and PKA substrate antibodies in the post-equatorial region **Chapter 4,** this was in contrast to the localisation of p55 in the tail fraction as revealed in **Chapter 5** using phospho (S/T) PKA substrate antibody. Therefore, calicin was excluded as a possible candidate for p55.

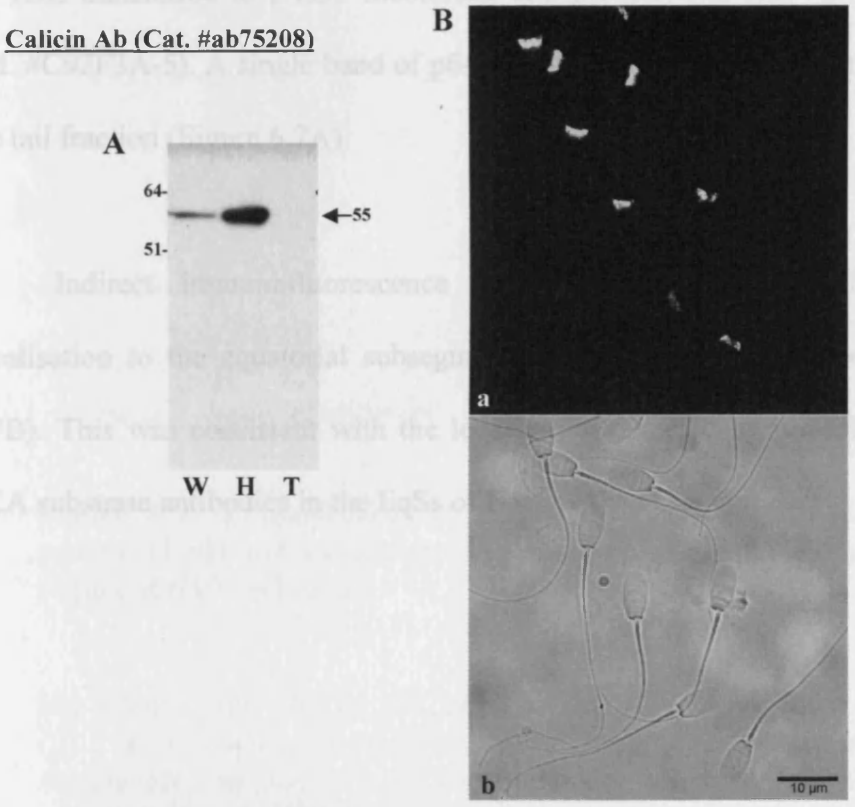

Figure 6.6: A protein of p55 was detected by immunoblotting and immunolocalisation to the post-equatorial region of the sperm head using calicin antibody.

(A) Non-capacitating (N) sperm solubilised and the supernatant was used for 1DE. Gel was transferred to membrane, blocked and probed with calicin Ab. **(B)** Immunolocalisation of calicin Ab. This experiment was replicated three times. **Ba** is a fluorescence only image and a paired image (fluorescence and light) is shown in **Bb** respectively. The results shown are representative of at least three experiments performed with different sperm samples.

6.4.5 HSP70/72 Ab detected p64 by immunoblotting and localised to the equatorial subsegment (EqSs) region of the sperm head

Based on the GeLC-MS protein identifications, HSP70 protein was a candidate for the pp64. Whole sperm (W), head (H) and tail (T) protein extracts were separated by 1DE transferred to PVDF membrane and probed with HSP70/72 Ab (Stressgen, Cat. #C92F3A-5). A single band of p64 was detected in the whole and head but not in the tail fraction (Figure 6.7A).

Indirect immunofluorescence of N sperm with anti-HSP70/72 showed localisation to the equatorial subsegment (EqSs) region of the sperm head (Figure 6.7B). This was consistent with the localisation detected by phospho (S/T) Akt and PKA substrate antibodies in the EqSs of N more than C sperm.

Hsp70/72 Ab (Cat.#C92F3A-5)

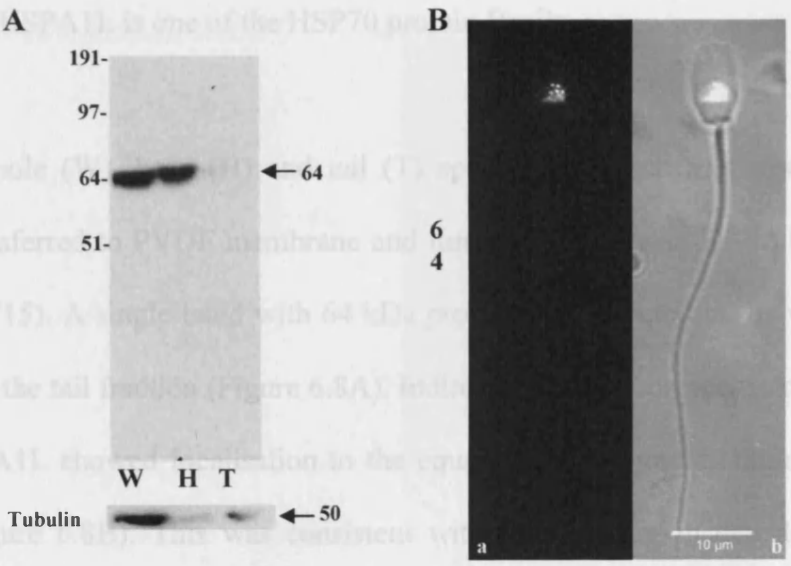

Figure 6.7: Immunoblotting with HSP70/72 antibody showed a protein of p64 and was localised to the equatorial subsegment (EqSs) region of the sperm head.

(A) Whole sperm (W) 2 x 10^6 , head fraction (H) 2 x 10^7 and tail fraction (T) 2 x 10^7. Whole sperm and the fractions were solubilised and the supernatant was used for 1DE. Gel was transferred to membrane blocked and probed with HSP70/72 Ab. **(B)** Immunolocalisation of HSP70/72 Ab. **Ba** is a fluorescence only image and a paired image (fluorescence and light) is shown in **Bb** respectively. The results shown are representative of at least three experiments performed with different sperm samples.

6.4.6 HSPA1L Ab detected p64 by immunoblotting and localised to the equatorial subsegment (EqSs) region of the sperm head

Heat shock 70 kDa protein 1-like (HSPA1L) protein was another candidate for the pp64. HSPA1L is one of the HSP70 protein family.

Whole (W), head (H) and tail (T) sperm protein extracts were separated by 1DE, transferred to PVDF membrane and immunoprobed with HSPA1L Ab (Abcam, Cat.#ab7715). A single band with 64 kDa protein was detected in the whole and head but not in the tail fraction (Figure 6.8A). Indirect immunofluorescence of N sperm with anti-HSPA1L showed localisation to the equatorial subsegment (EqSs) region of the head (Figure 6.8B). This was consistent with the localisation detected by phospho (S/T) Akt and PKA substrate antibodies to the EqSs of N more than C sperm. This result encouraged further investigation.

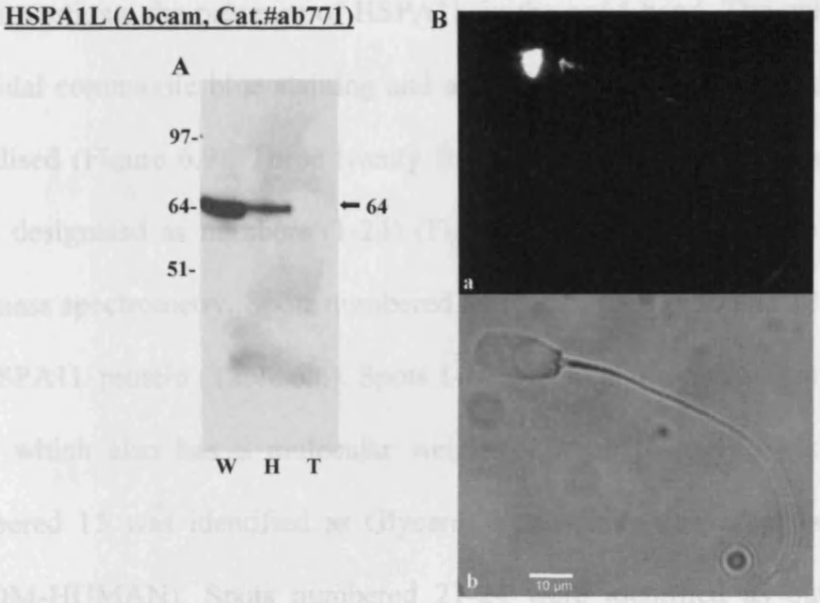

Figure 6.8: Immunoblotting with HSPA1L antibody showed a protein with p64 and was localised to the equatorial subsegment (EqSs) region of the sperm head.

(A) Whole sperm (W) 2 x 10^6 , head fraction (H) 2 x 10^7 and tail fraction (T) 2 x 10^7. Whole sperm and the fractions were solubilised and the supernatant was used for 1DE. Gel was transferred to membrane blocked and probed with HSPA1L Ab. **(B)** Immunolocalisation of HSPA1L Ab. **Ba** is a fluorescence only image and a paired image (fluorescence and light) is shown in **Bb** respectively. The results shown are representative of at least three experiments performed with different sperm samples.

6.4.7 HSPA1L protein was identified by 2DE in the 64 kDa spot excised corresponding to the pp64

The aim of this experiment was to separate the N sperm proteins by 2DE and then investigate the presence of HSPA1L in the pp64 band. The gel was stained with colloidal commassie blue staining and a number of spots of approximately p64 were visualised (Figure 6.9). These twenty four spots were manually excised. The protein spots designated as numbers (1-24) (Figure 6.9) were subjected to trypsin digestion and mass spectrometry. Spots numbered as 11, 12, 13, 14, 16 and 18 were all found to be HSPA1L protein (Table 6.6). Spots 1-10 were identified as serum albumin (ALBU-PIG) which also has a molecular weight of approximately 64 kDa whereas spot numbered 15 was identified as Glycerol-3-phosphate dehydrogenase, mitochondrial (GPDM-HUMAN). Spots numbered 21-24 were identified as outer dense fiber 2 (ODF2).

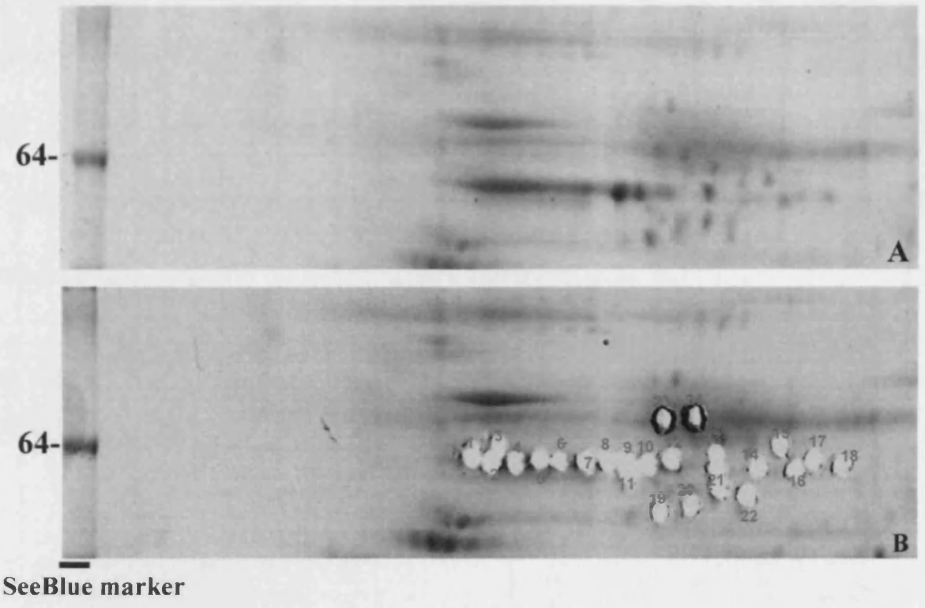

Figure 6.9: Two dimensional gel electrophoresis (2DE) of N sperm stained with colloidal commassie blue to aim to identify pp64.

(A) 3 x 10^6 N sperm are lysed with LDS sample buffer and 2DE was performed. See-Blue marker was used to excise the spots corresponding to p64 approximately. [N sperm was stained with colloidal commassie blue staining to confirm the presence of protein in the lysate]. **(B)** The highlighted protein spots were manually excised and identified by mass spectrometry. Numbers correspond to spot number in table 6.6.

Spot number	Protein name	accession number	Peptide sequence	Expect value	Top protein score	Top protein score C.I%
1	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	0.001	256	100
2	---	---	---	---	---	---
3	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	0.00029	238	100
4	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	2.4e-009	419	100
5	Serum albumin	ALBU-PIG		0.012	242	100
6	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	2.4e-008	231	100
7	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	2.1e-006	649	100
			YLYEIAR	0.00088		
8	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	1.1e-007	363	100
			YLYEIAR	0.016		
9	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	5.8e-008	599	100
			YLYEIAR	0.017		
10	Serum albumin	ALBU-PIG	**LGEYGFQNALIVR**	1.1e-005	362	100
11	---	---	---	---	---	---
12	Heat shock 70 kDa protein 1-Like	HS71L-PIG	**TTPSYVAFTDTER**	0.012	190	100
13	Heat shock 70 kDa protein 1-Like	HS71L-PIG	**TTPSYVAFTDTER**	5.2e-010	354	100
			ATAGDTHLGGEDFDNR	8.8e-006		
			LLQDYFNGR	0.013		
14	Heat shock 70 kDa protein 1-Like	HS71L-PIG	**TTPSYVAFTDTER**	6.8e-006	171	100
15	Glycerol-3-phosphate dehydrogenase, mitochondrial	GPDM-HUMAN	**LAFLNVQAAEEALPR**	6.1e-009	140	100
16	Heat shock 70 kDa protein 1-Like	HS71L-PIG	**TTPSYVAFTDTER**	5.1e-007	191	100
17	---	---	---	---	---	---
18	Heat shock 70 kDa protein 1-Like	HS71L-PIG	**TTPSYVAFTDTER**	0.00035	145	100
19	Outer dense fiber protein 2	ODF2-HUMAN	**LAECQDQLQGYER**	2.9e-006	246	100
			GHLQAQLR	0.036		
20	Outer dense fiber protein 2	ODF2-HUMAN	**LAECQDQLQGYER**	3.1e-007	313	100
			GHLQAQLR	0.037		
21	Outer dense fiber protein 2	ODF2-HUMAN	**LAECQDQLQGYER**	1.1e-008	352	100
			GHLQAQLR	0.02		
22	Outer dense fiber protein 2	ODF2-HUMAN	**LAECQDQLQGYER**	.0012	156	100
23	Outer dense fiber protein 2	ODF2-HUMAN	**LAECQDQLQGYER**	0.02	131	100
24	---	---	---	---	---	---

Table 6.6: A list of proteins identified by mass spectrometry from the 2DE spots shown in figure 6.9.

N sperm were subjected to two dimensional electrophoresis (2DE) and the gel was stained with colloidal commassie blue (Figure 6.9). Twenty four spots were manually excised corresponding to the 64 kDa molecular weight of the SeeBlue marker trypsin digested and subjected to MS/MS analysis. Successful identifications were obtained for 21 spots.

6.4.8 Immunoprecipitation (IP) using the phospho (S/T) PKA substrate antibody

The identification of HSPA1L from multiple 2D bands supported the concept of post translational modifications to this protein. The aim of this experiment was to immunoprecipitate proteins with the phospho (S/T) PKA substrate antibody to investigate if the pp64 is HSPA1L detected with this antibody. N and C sperm proteins were solubilised and immunoprecipitated with the phospho (S/T) PKA substrate Ab. The proteins were immunoblotted with phospho (S/T) PKA substrate Ab. The IP with phospho (S/T) PKA substrate Ab detected pp97, pp96, pp90 and pp64, albeit very weakly. However, pp55 was not detected perhaps due to a loss of the phosphorylation during the IP (Figure 6.10).

To investigate whether HSPA1L could be the pp64, the same membrane in (Figure 6.10) was stripped and probed with HSPA1L Ab (Abcam, Cat.#77155) (Figure 6.11). A band corresponding to HSPA1L was detected in protein lysates and in the immunoprecipitates. However, HSPA1L was also precipitated by beads alone in the absence of the phospho (S/T) PKA substrate Ab. This suggested that the precipitation was not specific for the phospho PKA substrate antibody. The lack of specificity makes it impossible to confirm HSPA1L as pp64. However, the possibility cannot be excluded at this time either. The investigation of HSPA1L as a dephosphorylated protein in C sperm merits further investigation using alternative precipitation methods or phosphopeptide mapping by mass spectrometry.

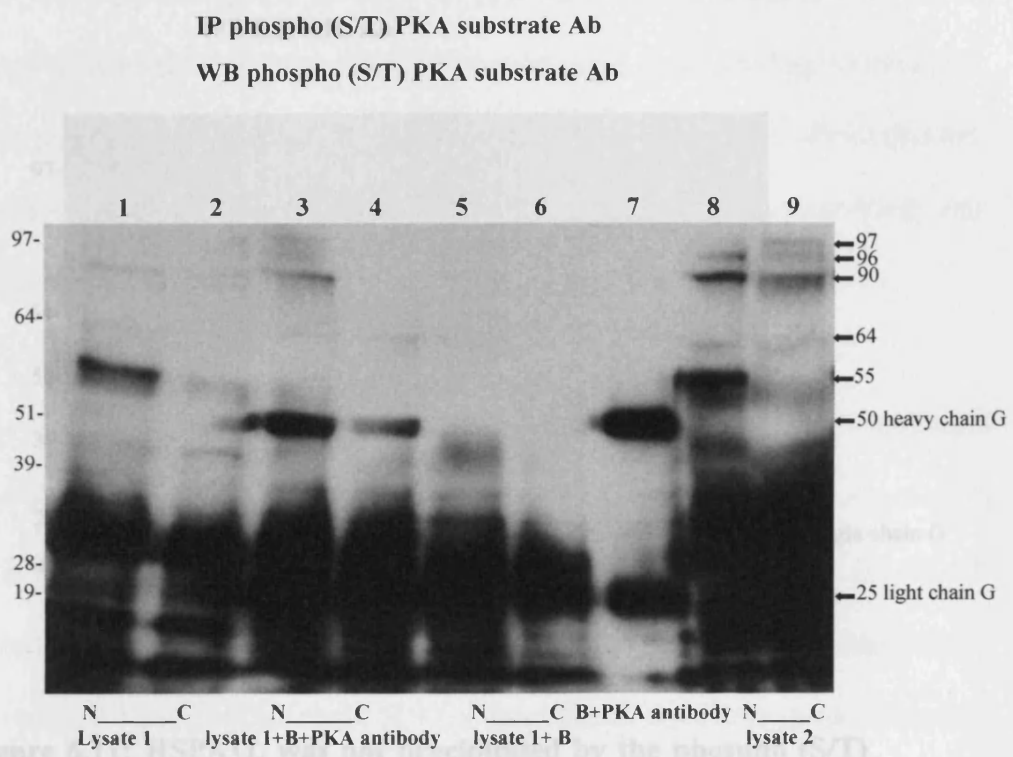

Figure 6.10: Immunoprecipitation of pp97,pp96 and pp90 but not pp55 and pp64 using phospho (S/T) PKA substrate antibody.

Boar sperm were incubated under non-capacitating (N) or capacitating (C) conditions for 1 h. Following incubation sperm were solubilised and the lysates incubated with phosphatase and protease inhibitors (lysate 1) for 1 h at 4 °C with Protein G Dynabeads (B) conjugated with phospho (S/T) PKA substrate Ab. The beads were then washed, boiled and the immunoprecipitates resolved using 1DE followed by immunoblotting with phospho (S/T) PKA substrate Ab overnight. Lanes 1 and 2: beads incubated with N or C lysates with phosphatase and protease inhibitors only. Lanes 3 and 4: the precipitated proteins of N and C lysates with beads (B) and phospho (S/T) PKA substrate antibody. Lanes 5 and 6: N and C lysates incubated with beads (B). Lane 7: antibody conjugated beads. Lanes 8 and 9: N and C sperm lysate without phosphatase or protease inhibitors (lysate 2). The results shown were repeated at least 3 times with different sperm samples.

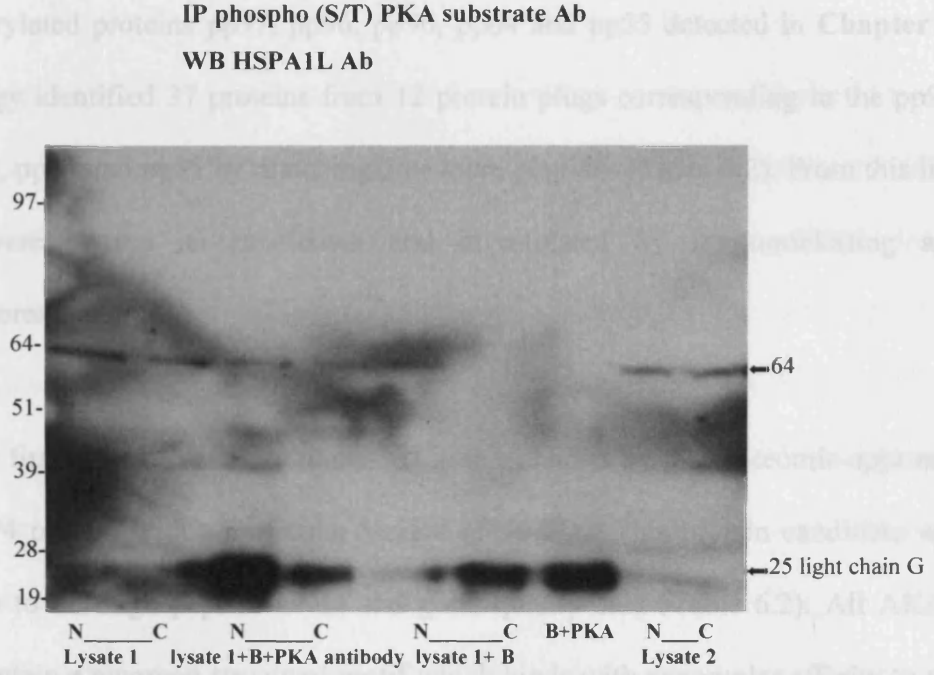

Figure 6.11: HSPA1L was not precipitated by the phospho (S/T) PKA substrate antibody.

The membrane in **Figure 6.10** was stripped, blocked and reprobed overnight with HSPA1L antibody. Membrane was washed with BPS and probed with anti-mouse secondary Ab for 1 h. The results shown were repeated at least 3 times with different sperm samples.

6.5 Discussion

The aim of this chapter was to try to use GeLC-MS to identify the dephosphorylated proteins pp97, pp96, pp90, pp64 and pp55 detected in **Chapter 4**. This strategy identified 37 proteins from 12 protein plugs corresponding to the pp97, pp96, pp90, pp64 and pp55 by matching 2 or more peptides (Table 6.2). From this list, proteins were chosen as candidates and investigated by immunoblotting and immunofluorescence.

The first protein candidate identified in this chapter by the proteomic approach was AKAP4 protein with a molecular weight of 96 kDa. This protein candidate was chosen due to the high peptide count and good quality data (Table 6.2). All AKAP proteins contain a common structural motif which binds with nanomolar affinity to the regulatory subunit of PKA (Carr et al., 1992; Carr et al., 1991). These subunits bind to the AKAP proteins and regulate sperm functions such as motility. Therefore it was encouraging to choose this protein as a candidate for pp97, pp96 and pp90 detected by the phospho (S/T) PKA substrate antibody in C sperm.

AKAP4 is the major fibrous sheath protein and is the product of an X-linked gene (Moss et al., 1997) expressed only in spermatids (Carrera et al., 1994). It was demonstrated that the AKAP protein RI and RII domains bind with PKA to recruit PKA to the fibrous sheath which phosphorylates neighbouring proteins that regulate flagellar function (Miki and Eddy, 1998; Miki and Eddy, 1999). Therefore, AKAP4 is a scaffold for localising and grouping functionally important proteins including PKA. The importance of AKAP4 protein in sperm motility was investigated by targeting the AKAP4 gene (Miki et al., 2002). This study demonstrated that mutation of the AKAP4

gene disrupts the motility of mouse sperm and causes infertility due to the effect on the signal transduction and glycolytic enzymes necessary for flagellar function.

In this chapter, three proteins (p96, p64 and p55) were detected by the AKAP4 Ab. These proteins were the same molecular weight as the pp96, pp64, and pp55 detected by phospho (S/T) PKA substrate Ab but not pp90 which was the fourth protein detected in **Chapter 4**. The localisation of these proteins in intact boar sperm incubated under N or C conditions are to the principal piece of the flagellum. This localisation was reported in previous studies such as in bovine sperm (Vijayaraghavan et al., 1997b). To date, there are no studies on AKAP4 in boar sperm. Although AKAP4 was a strong candidate for pp97, pp96 and pp90 detected in **Chapter 4,** it is localised to the principal piece of boar sperm. This was in contrast to the localisation of these proteins detected by phospho (S/T) PKA substrate Ab mainly in the sperm head **(Chapter 4)**. Moreover, the localisation of pp96 and pp64 was in the sperm head fraction and not in the tail fraction **(Chapter 5)**. This protein was therfore excluded as a possible candidate for the dephosphorylated protein.

The second protein candidate identified was AKAP3 which is also formally designated as AKAP110 because it migrates at approximately 110 kDa in SDS-PAGE. It is a sperm specific protein and a member of the AKAP family. Immunocytochemical analysis of its subunits has reveald that R1 α and RI β are predominantly localised to the acrosomal segment of the head whereas RIIα is confined in the sperm flagellum (Vijayaraghavan et al., 1997b). AKAP3 functions as an anchoring protein for the subcellular localisation of PKA in the fibrous sheath(Moss and Gerton, 2001).

We identified AKAP3 by immunoblotting and immunofluorescence using two different AKAP3 antibodies. The first antibody (AKAP3, Santa Cruz) detected major bands by immunoblotting (Figure 6.4) and in parallel to that immunofluorescence showed fluorescence in the anterior acrosome region called the apical ridge of boar sperm (Figure 6.4). This localisation was similar to that reported in bovine sperm (Vijayaraghavan et al., 1999). No previous study has reported on the localisation or immunoblotting of AKAP3 in boar sperm. Although the immunoblotting of AKAP3 was unsatisfactory due to non-specific binding to boar sperm proteins, the fluorescence in the apical region of the sperm head was similar to that detected by phospho (S/T) PKA and Akt substrate antibodies **(Chapter 4)**. Therefore, an alternative AKAP3 antibody was used in order to detect AKAP3.This antibody (AKAP3, Abcam) was expected to detect a protein with molecular weight of 95 kDa but three proteins of p96, p64, and p55 were detected by immunoblotting (Figure 6.5). Immunofluorescence of N boar sperm showed localisation of AKAP3 in the principal piece of the tail by this antibody (Figure 6.5). This localisation and immunoblotting data is similar to that detected by AKAP4 Ab **(Section 6.4.2)**. This similarity may be due to the peptide homology between these two proteins and suggested that they may share common function (Vijayaraghavan et al., 1999). Despite the fact that the two AKAP3 antibodies were unable to detect AKAP3 by immunoblotting, AKAP3 is still a possible candidate for the pp97, pp96 and pp90. This candidate deserves more investigation by carefully using a better quality antibody that can detect only AKAP3 proteins. However, no such antibody exists currently and would be made.

The third protein candidate investigated in this study was calicin (CALI), which is a p55. This is a basic cytoskeletal protein localised in the calyx in the perinuclear

theca of the sperm head (Fawcett, 1975; von Bulow et al., 1995). The calyx tightly surrounds the posterior part of the nucleus to maintain the sperm head architecture during spermatogenesis and fertilisation. This protein is sperm specific and encoded by a 2.2 kb mRNA detected only in the testis (von Bulow et al., 1995). CALI Ab was used to detect this protein in N boar sperm by immunoblotting and this showed a single band at p55 (Figure 6.6) and immunolocalisation was in the postequatorial region of boar sperm (Figure 6.6). The localisation of CALI protein to the post-equatorial region was previously reported in boar sperm head with 60 kDa protein detected by immunoblotting (Lecuyer et al., 2000) and in bovine sperm head (von Bulow et al., 1995). These results were consistent with pp55 protein detected by both phospho (S/T) PKA and Akt substrate antibodies.in addition that antibodies revealed fluorescence in the post-equatorial region of these proteins in N boar sperm **(Chapter 4)**. In contrast, pp55 was detected in the tail fraction only by phospho (S/T) PKA substrate antibody **(Chapter 5).** Therefore, CALI was excluded as a candidate for pp55.

The final proteins identified in this study as a candidate for pp64 were isoforms of Heat shock protein 70 (HSP70/72) and heat shock 70 protein1-like (HSPA1L). These two proteins are members of the heat shock protein 70 family which are produced by several distinct genes and named according to the approximate relative molecular mass of their encoded proteins (Brocchieri et al., 2008). Heat shock proteins are chaperones essential for folding the newly synthesised proteins to avoid aberrant interactions and to achieve the correct conformation. These proteins increase in the cells in response to the elevations in temperature, chemical or physical stress and viral infection to protect important cell proteins from denaturation and aggregation (Beere, 2004). The function of this protein is to maintain the proper protein conformation,

stabilising unfolded proteins prior to their assembly into complexes, protein trafficking and translocation through membranes (Gething and Sambrook, 1992).

HSP70 is synthesised during spermatogenesis because ejaculated sperm are transcriptionally inactive and their translational machinery is lost from the residual cytoplasm. Hence, stress-induced synthesis of HSP70 in sperm is not possible and the sperm is dependent upon pre-formed HS70. Bedside their protective functions against stresses, HSPs have been recently indicated as important factors for sperm fertilising ability (Matwee et al., 2001; Spinaci et al., 2005). Several HSP family proteins are expressed in the mammalian testis and sperm. HSP70 was identified in male germinal cells during spermatogenesis in mouse, rat, bull, boar and humans (Miller et al., 1992). HSP70 has been recently found to reduce the fertilisation rate by binding to anti-HSP70 suggesting an important role of this protein during sperm-oocyte interaction (Matwee et al., 2001; Spinaci et al., 2005). Intriguing studies on other heat shock proteins have implicated them in key roles at fertilisation (Ecroyd et al., 2003).

In this chapter, two heat shock 70 proteins (HSP70 and HSP71L) were identified. Both proteins are expressed in the testis and HSP70/72 has been shown to be necessary for the progression of meiosis in mouse germ cells (Eddy, 1999). On the other hands HSP71L protein is mainly expressed in spermatids (Vos et al., 2008) and HASP1L is not influenced by heat (Ito et al., 1998). In this study, both heat shock proteins were detected in whole and head N spermfractions and were localised to the EqSs in the sperm head. In contrast to the sperm heads, tail fractions showed no presence of these proteins. In **Chapter 4** the immunolocalisation of phospho (S/T) PKA and Akt substrate antibodies was more in N than C cells in the EqSs and this was

similar to that shown by HSP70/72 and HSP71L antibodies in **Chapter 6**. This localisation of HSP70 in the EqSs has also been reported in N boar sperm (Spinaci et al., 2005; Volpe et al., 2008). Interestingly, the EqSs is functionally important as a site of fusion with the egg membrane in eutherians mammals.

Finally, The HSPs (HSP70 and HSP71L) results were encouraging to be validated by further investigation to confirm these identifications using both 2DE and immunoprecipitation. N sperm was separated by 2DE analysed by proteomic approaches to confirm the identification of HSP70 and HSPA1L proteins in the p64 protein in the spots picked from the gel. Immunoprecipitation experiments were performed to investigate whether pp64 is the HSPA1L protein. N sperm proteins were incubated with phospho (S/T) PKA substrate antibody to precipitate pp97, pp96, pp90, pp64 and pp55. This approach was only able to precipitate the higher molecular weight proteins (pp97, pp96 and pp90) but not pp55 compared with the lysate 1 and beads. This may be due to the loss of phosphorylation during the long process of immunoprecipitation. pp64 was detected but was similar to that seen with the lysate 1 and beads which meant that it was not precipitated by the phospho (S/T) PKA substrate antibody but bound to the beads. Immunoblotting of the precipitated phosphorylated proteins with HSPA1L showed a pp64 band similar to that seen with the lysate 1 and beads.

Taken together, results from this chapter suggested that AKAP4 protein candidate was not pp97, pp96 and pp90 detected by phospho (S/T) PKA and Akt substrate antibodies in **Chapter 4** because of the localisation in sperm tails. AKAP3 protein was not confirmed to be pp97, pp96 and pp90 due to the relatively poor quality

of the AKAP3 antibodies used to detect AKAP3 proteins by immunoblotting. Despite the fact that calicin was a strong candidate for pp55 due to the high peptide count and the molecular weight (55 kDa), the localisation in the tail fraction in **Chapter 5** indicated that this protein was not the pp55. On the other hand, HSP70 proteins were good candidates for pp64. Unfortunately, the immunoprecipitation have not given a definitive result for pp64. Thus, it is not possible to exclude the heat shock proteins as candidates nor it is possible to say that any dephosphorylated proteins are HSP70 family members. In order to do this, an improvement in immunoprecipitation would be required. Alternatively, the use of phosphoproteomic techniques could help in the identification of the phosphoproteins characterised in the earlier chapters.

CHAPTER 7

General discussion

S/T and tyrosine protein phosphorylation occurs in sperm during capacitation but only a few phosphorylated proteins have been identified (Carrera et al., 1996; Ecroyd et al., 2003; Ficarro et al., 2003; Mandal et al., 1999; Naaby-Hansen et al., 2002). The processes regulated by protein phosphorylation include capacitation, hyperactivated motility and the AR, all of which are required for sperm to reach and fertilise the egg. Signalling by protein phosphorylation in sperm capacitation is still poorly understood. Although protein tyrosine phosphorylation is less frequent than protein S/T phosphorylation in biology, more studies have investigated the phosphorylation on tyrosine residues of sperm proteins during capacitation than that of S/T phosphorylation. Therefore, the main aim of this thesis was to investigate and characterise the changes in phospho (S/T) proteins in boar sperm incubated under C conditionsusing immunoblotting and immunofluorescence.

The first success of my project was to establish a boar sperm model system to study signalling events during capacitation. I studied movement, viability and protein tyrosine phosphorylation, which increased during capacitation. This endeavour ensured that the model system was robust and made the observation of a loss of protein S/T phosphorylation a reliable phenomenon as this result was a surprise when I first observed it. The fact that I saw an increase in tyrosine phosphorylation while seeing a decrease in S/T phosphorylation meant that it was unlikely to be an artefact. Rather, it occurred at the same time during incubation under C conditions and was reproducible.

For the first time, multiple S/T dephosphorylation events that occur in a bicarbonate dependent fashion were identified in this study in boar sperm in the first hour of incubation (Figure 7.1B). Different phospho (S/T) kinase substrate antibodies

were used and dephosphorylation of six S/T phosphoproteins were observed in C sperm compared with N cells. This has not been previously observed in any other mammalian species. There are only very few reports on S/T phosphorylation of sperm proteins during capacitation. One of these studies investigated the S/T protein phosphorylation of human sperm during capacitation (O'Flaherty et al., 2004) used a different PKA substrate antibody motif R-X-X-S/T to the PKA substrate antibody motif R-R-X-S/T used in this study. S/T phosphorylation was reported in human sperm proteins incubated under C conditions and localised along the flagellum. These results are different from the changes of S/T proteins reported in this thesis using boar sperm. This study identified a more complex change in protein phosphorylation than previously proposed.

The involvement of PKA during capacitation has been demonstrated to be activated directly by cAMP or through inhibition of phosphodiesterases (Figure 7.1A), for example 3-isobutyl-1-methylxanthine (IBMX) (Leclerc et al., 1996; Visconti et al., 1995b). Further investigation has been performed in this study to determine the effect of cAMP on bicarbonate-dependent dephosphorylation of S/T sperm proteins. Sperm incubated in N medium containing the cAMP analogue dibutyryl cAMP (dbcAMP) and IBMX did not show protein dephosphorylation. In contrast, incubation in C medium with dbcAMP/IBMX showed the usual dephosphorylation of pp97, pp96, pp90, pp64 and pp55 as well as increased phosphorylation of other proteins (pp68, pp51 and pp29). The increase of phosphorylation has been reported previously and is consistent with that reported in boar sperm incubated with cAMP analogue cBiMPS (Harayama, 2003) and mouse sperm (Goto and Harayama, 2009). This phosphorylation was localised in the midpiece and principal piece of the sperm

flagellum which may suggest a role in sperm motility. Consistent with this, the addition of dbcAMP/IBMX to the sperm incubated under C conditions showed an increase of phosphorylation on tyrosine residues of pp190, pp100, pp96, pp92, pp70. This was consistent with the previously reported study in boar (Harayama et al., 2004b) and several other species for example in mouse sperm cells (Visconti et al., 1995b), bull sperm cells(Galantino-Homer et al., 1997) and human sperm cells (Leclerc et al., 1996). The increased S/T and tyrosine phosphorylation in sperm incubated under C conditions with dbcAMP/IBMX was shown to be localised in the sperm tail only. This localisation of S/T phosphorylated proteins to the tail may reveal a possible effect on the sperm motility as was reported in boar (Harayama and Nakamura, 2008). Thus, the S/T protein dephosphorylation under C conditions reported in this thesis is non cAMP-dependent (Figure 7.1B).

In order to investigate the possible cause of the S/T dephosphorylation in boar sperm proteins in this study, cells were incubated with the phosphatase inhibitor calyculin A. This S/T phosphatase inhibitor prevented the dephosphorylation of pp96, pp90, pp64 and pp55 but not pp105. Based on this data, we conclude that two pathways of protein dephosphorylation are active during capacitation and independent of cAMP (Figure 7.1B). More recently, a study has reported that mouse sperm incubated with calyculin A become hyperactivated due to the suppressive effect of this inhibitor to the PP1/PP2 phosphatases and subsequently increased phosphorylation of flagellar proteins (Goto and Harayama, 2009).

Indirect immunofluorescence results have shown that the phosphorylation on S/T proteins was more intense in N than C sperm heads using different phospho (S/T)

kinase substrate antibodies. Therefore, subcellular fractionation of sperm heads and tails was performed for the localisation of the dephosphorylated proteins. This was achived by using sonication and differential density gradient centrifugation. The purification of sperm head and tail fractions was determined by two main methods. The first was by monitoring each sonicated boar sperm fraction under light microscopy to confirm that head and tail sperm were completely detached and pure. The second method used to assess the purity of heads and tail fractions was by using specific antibodies for each fraction. The purified head and tail fractions were solubilised and 1DE gel was transferred to membranes. Immunoblotting reported that pp97, pp96 and pp64 are head proteins while pp90 and pp55 are tail proteins detected by phospho (S/T) PKA substrate antibody. This localisation may help to identify these proteins and indicate their possible function during boar sperm capacitation. The immunolocalisation with this antibody in intact boar sperm showed that phospho (S/T) proteins were localised in the apical ridge, acrosome, equatorial subsegment (low levels), post equatorial region, posterior ring and flagellum (low levels) in 1 h N sperm. No phosphorylation was observed in 1 h C sperm in the apical ridge and equatorial subsegment. This observation suggested that the dephosphorylated proteins may localise in the head sperm more than tails. However, the subcellular fractionation results did not support this suggestion as only pp97, pp96 and pp64 are head sperm proteins and pp90 and pp55 are tail proteins.

The final aim of this study was to identify the dephosphorylated proteins detected by both phospho (S/T) PKA and Akt substrate antibodies. In order to achieve this, a combination of approaches was used including advanced proteomic analysis (GeLC-MS), immunoblotting and immunoprecipitation. GeLC-MS of boar sperm

identified 37 proteins. Five proteins were chosen as candidates for the dephosphorylated proteins: AKAP4, AKAP3, CALI and HSP70 and HSPA1L. These five candidates were chosen based on the GeLC-MS analysis including the molecular weight which was similar to that of S/T dephosphorylated proteins, as the number of peptide counts and the quality of the MS data. Importantly, some of these candidates were also chosen due to the immunolocalisation of these candidates in sperm similar to that observed in boar sperm detected by the phospho (S/T) PKA substrate antibodies **Chapter 4**.

Immunoblotting of the five protein candidates of the dephosphorylated proteins was performed using specific antibodies. p96, p90 and p55 were detected by AKAP4 Ab and localised to the principal piece of the sperm tails. This is in contrast to the localisation of the pp96 in the head. Therefore AKAP4 was excluded as one of the possible pp97 and pp96 candidates. AKAP3 was another candidate for these proteins. Immunoblotting using AKAP3 Ab showed non-specific binding to the sperm proteins but IIF results showed that AKAP3 proteins were localised to the apical ridge of the sperm heads. This immunolocalisation was very encouraging because it was reported by the phospho PKA and Akt substrate antibody in N sperm. Therefore, an alternative AKAP3 antibody was used in order to obtain better immunoblotting results. Immunolocalisation of AKAP3 using AKAP3 Ab detected fluorescence in the principal piece of the sperm tails. This is similar to the AKAP4 detected with AKAP4 Ab. This similarity may be due to the fact that some peptides are shared between AKAP3 and AKAP4 in pig sperm as they are in the same protein family. Whilst AKAP3 is still a possible candidate for pp97, pp96 and pp90 a better quality antibody and further study is needed to potentially achieve identification. The last two proteins

investigated were HSP70 and HSPA1L which were considered as possible candidates for pp64. HSP70/72 and HSPA1L antibodies detected p64 by immunoblotting which was localised to the equatorial subsegment region of the sperm head. This was consistent with the localisation detected by Akt and PKA antibodies. Therefore HSPA1Lwas considred to be a strong candidate the pp64 and warranted more investigation to achieve definitive identification.

Although five protein candidates were chosen from the GeLC-MS analysis to be investigated in order to identify the dephosphorylated proteins detected in **Chapter 4**, only HSPA1L was a strong candidate for pp64 and we aimed to validate this by immunoprecipitation. This approach was unable to precipitate the lower molecular weights of targeted dephosphorylated proteins which may be due to the efficiency of the phospho (S/T) PKA substrate antibody in the or due to the loss of phosphorylation because of long process of immunoprecipitation which about 3 h. Evendough, HSPA1L is strong candidate for pp64 and modification of immunoprecipitation could help identify it.

Summary of main achievements of the thesis

The data presented in this study has led to a proposed model for bicarbonate-dependent (S/T) protein dephosphorylation in boar sperm (7.1B) and concluded the following:

- A model system for investigating early changes (up to 1 h) of signalling transduction pathways in boar sperm incubated under capacitating conditions was established.
- Analysis of protein phosphorylation revealed bicarbonate-dependent S/T dephosphorylation of multiple proteins in boar sperm.
- Bicarbonate-dependent S/T protein dephosphorylation was cAMP-independent.
- Calyculin A inhibited S/T protein dephosphorylation of all proteins but not pp105 which revealed two distinct pathways, with one regulated by PP1 and/or PP2A downstream of the bicarbonate.
- Good quality immunofluorescence figures have localised a number of proteins in boar sperm cells (Figure 7.2)
- Identifications of the S/T dephosphorylated proteins were challenging and no definitive identification was achieved due to technical difficulties (choice of proteins), quality of the antibodies used or immunoprecipitation protocol issues.

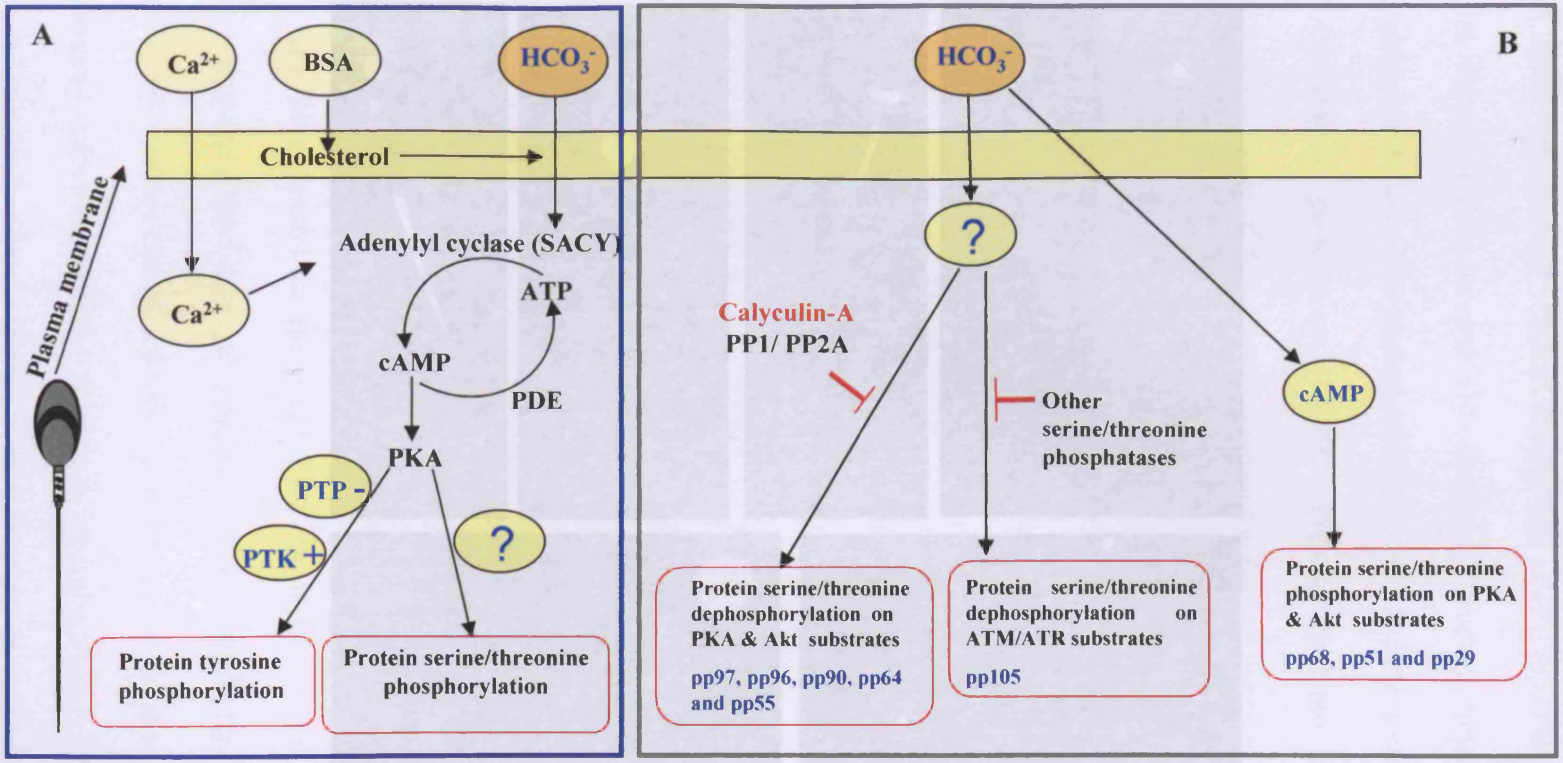

Figure 7.1: Proposed pathways of protein phosphorylation and dephosphorylation in sperm during capacitation.

(A) Proposed pathway of tyrosine and serine/threonine (S/T) protein phosphorylation pathways in sperm during capacitation by Visconti et al. (2001). **(B)** ***Proposed pathway of serine/threonine (S/T) protein dephosphorylation pathways in boar sperm during capacitation reported in this thesis***. Our data shows that bicarbonate may stimulate PP1/PP2A which causes S/T dephosphorylation of PKA and Akt substrates during capacitation. Addition of calyculin A inhibited PP1/PP2A which prevented dephosphorylation of S/T PKA and Akt substrates during capacitation. Bicarbonate induces the dephosphorylation of S/T ATM/ATR substrates under capacitating conditions. This is not inhibited by calyculin A and hence may be caused by other phosphatases.

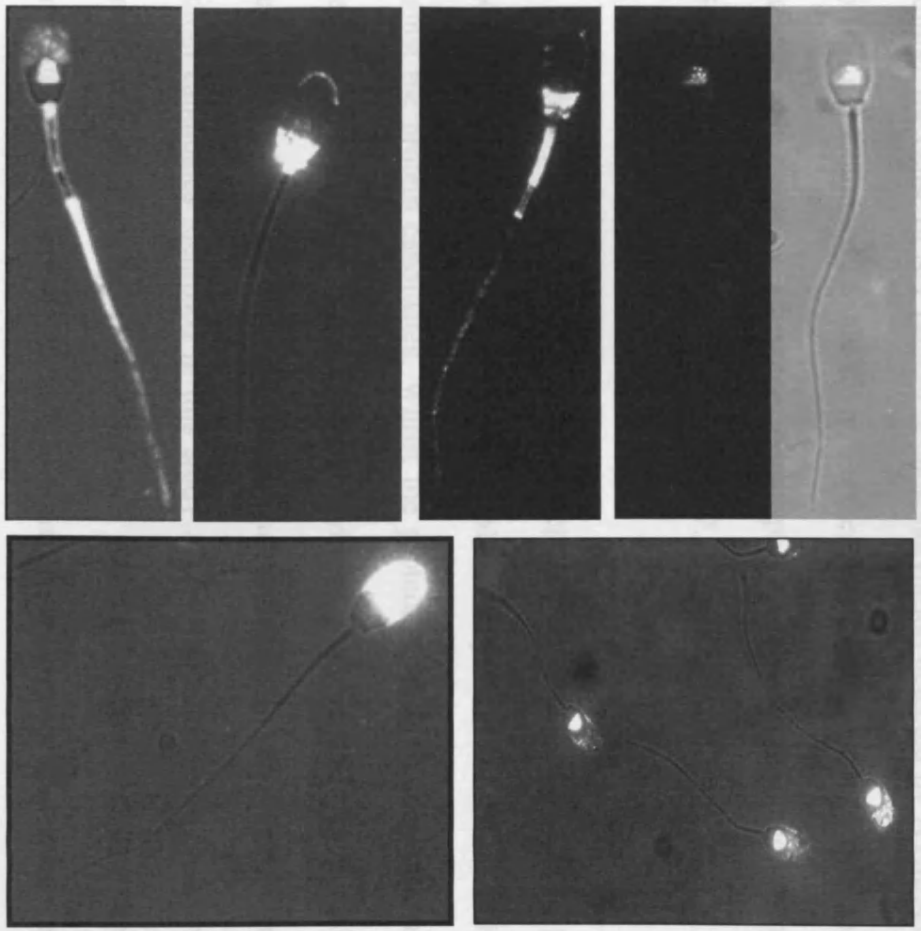

Figure 7.2: Indirect immunofluorescence figures of sperm cells represent the good quality data generated throughout the thesis.

Future research

This study successfully detected for the first time multiple proteins dephosphorylated on S/T residues during boar sperm capacitation. Moreover, the cause of the dephosphorylation was also detected by using calyculin A which is a S/T phosphatase inhibitor. The phosphatases responsible for dephosphorylation of the PKA and Akt substrate proteins are PP1/PP2 which are the main targets for calyculin A. In addition, the cause of S/T protein dephosphorylation detected by ATM/ATR substrate Ab was not examined in this study. Further investigation is needed to more carfully elucidate this pathway of protein dephosphorylation during capacitation maybe by using other phosphatase inhibitors (Figure 7.1B).

Finally, we aimed to identify the dephosphorylated proteins pp97, pp96, pp90, pp64 and pp55 using proteomic analysis followed by immunoblotting with antibodies against the protein candidates. Whilst the proteomic analysis identified a number of possible candidates of the dephosphorylated proteins the immunoblotting and immunoprecipitation approaches were challenging with some proteins. Of these protein candidates identified in this study AKAP3 remains a candidate but different and better quality antibodies are required. HSP71L protein was also identified as a candidate for pp64. Immunoblotting and immunofluorescence have supported this but we were unsuccessful in the validataion by immunoprecipitation. Therefore, this protein remains a candidate and modifying the immunoprecipitation method used, 2DE or other approaches are all options. This thesis has demonstrated the difficulties in achieveing definitive identification of a phosphoprotein. In the future a range of phosphoproteomic approaches may be important to achieve identifications of such proteins.

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APPENDICES

Appendix I: Tyrode's: Incubation medium for the capacitating (C) and non-capacitating (N) medium

To keep the osmolality equal in both C and N buffer, the two buffers were initially prepared without NaCl and $NaHCO_3$ as following:

component	recipe	add for 100 ml		final conc
		Capacitation	Non-capacitation	
NaCl	2.922 g/100 ml	20.4 ml	23.2 ml	(90-)120 mM
Hepes (pH 7.58)	1.192 g/25 ml	10.0 ml	10.0 ml	20 mM
Glucose	900 mg/10 ml *(fresh)*	1.0 ml	1.0 ml	5.0 mM
KCl	3.728 g/100 ml	0.62 ml	0.62 ml	3.1 mM
$MgSO_4.7H_2O$	247 mg/10 ml	0.4 ml	0.4 ml	0.4 mM
Lactate	stock=60% *(fresh)*	0.31 ml	0.31 m	21.7 mM
$NaH_2PO_4.2H_2O$	138 mg/10 ml	0.3 ml	0.3 ml	0.3 mM
Kanamycin	dry powder *(fresh)*	10 mg	10 mg	100 □g/ml
$NaHCO_3$	2.100 g/100 ml *(fresh)*	6.0 ml	XXXXX	15 - 0 mM
pyruvate	22 mg/ml *(fresh)*	0.5 ml	0.5 ml	1 mM
$CaCl_2.2H_2O$	1.470 g/10 ml	0.2 ml	0.2 ml	2.0 mM
BSA	300 mg/ml *(fresh)*	1 ml	1 ml	3 mg/ml

All components were mixed then divided into two volumes one for C buffer and the other for N buffer. 20.4 ml NaCl and 6 ml $NaHCO_3$ were added to the C buffer. 23.2ml NaCl was added to the N buffer. Each buffer was made up to a final volume of 100 ml with deionised water. Osmolality confirmed to be between 295-300 mOsm/kg and pH 7.40 ± 0.05. The N buffer was placed in air tight sealed tube whereas the C buffer was also placed in a tube but with the screw cap (top) removed and left overnight in the incubator at 38.5°C and 5 % CO_2 prior to use overnight.

Appendix II: Preparation of solutions for Percoll density gradient centrifugation

10X M medium

Chemical	50 ml	250 ml
NaCl (MW 58.44)	4.003 g	20.016 g
KCl (MW 74.56)	0.093 g	0.466 g
Hepes (MW 238.3)	2.383 g	11.915 g
Glucose (MW 180.16)	0.901 g	4.504 g
Kanamycin	5mg	25mg

1X M medium

5 ml 10X stock was diluted with 45 ml water.

Final concentration of 137 mM NaCl; 2.5 mM KCl; 20 mM Hepes; 10 mM glucose;

pH 7.55. 90 μg/ml kanamycin was added (4.5mg/50ml)

Isotonic Percoll (285-300mOsm)

12:1 Percoll: 10X M medium (mix 48 ml Percoll with 4 ml 10X M medium)

35 % and 70 % Percoll

70%: 28 ml 100% Percoll + 12 ml 1X M medium

35%: 14 ml 100% Percoll + 26 ml 1X M medium

Percoll solutions were placed in the incubator (38.5 ^{o}C) overnight to warm.

Tris buffer sucrose solution (TBSS)

0.005 M Tris (0.303 g) + 0.25 M sucrose (22.52 g)

50 mg Kanamycin was added to 500 ml H_2O

Appendix III: Sperm preparation using Percoll density gradient centrifugation

1. 15 ml (70%) Percoll was put in the bottom of 2 x 50 ml Falcon tubes each
2. 15ml (35%) Percoll was carefully layered on the top without disturbing the interface between the 35% and 70% fractions.
3. 15 ml semen was layered on the top.
4. Spun 755 *g* for 20 minutes, brake off.
5. All solutions were removed except the pellet at the base of the 70% fraction.
6. One pellet (one tube) was resuspended in 10 ml 1X M buffer and the sperm were counted.
7. 10 ml 1X M buffer was into two tubes (one for C and the other for N) in each (10^8 cells).
8. The two tubes were spun at 500 *g* for 5 min, and then the supernatant was removed.
9. The two pellets were resuspended in 10 ml C buffer and the other in 10 ml N buffer.
10. The N buffer is incubated in air tight sealed tube while the C buffer should also be placed in a tube but with the screw cap (top) in the incubator at 38.5°C and 5 % CO_2.

Appendix IV: Preparation of immunoblotting buffers

Preparation of PBS-Tween:

500 ml H_2O

5 tablets PBS.

1 ml Tween-20.

IBT:

0.2% (1g in 500ml) I-Block

0.1 %(0.5ml in 500ml) Tween-20

0.4% (2g in 500ml) Sodium azide.

This solution was made in the following order: PBS (5 tablets) was added to 500ml water and dissolved. The PBS was preheated to 80 °C then 1g of "I Block" powder was added and dissolved. The buffer was Cooled and sodium azide was then added.

Appendix V: a copy of the published work of this thesis

Alnagar, F. A., Brennan, P. and Brewis, I.A (2010). Bicarbonate-dependent serine/threonine protein dephosphorylation in capacitating boar spermatozoa. *J Androl* **31**, 393-405.

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Bicarbonate-Dependent Serine/Threonine Protein Dephosphorylation in Capacitating Boar Spermatozoa

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ABSTRACT: This study investigates the dynamics of serine/threonine (S/T) protein phosphorylation in sperm incubated under capacitating (C) conditions using the boar as a model system. For the first time, this approach has identified multiple dephosphorylation events that occur in a bicarbonate-dependent fashion. Different phospho-(S/T) kinase substrate antibodies were used, and dephosphorylation of 5 S/T phosphoproteins was observed in C sperm compared with noncapacitated (N) cells. Specifically, dephosphorylation of 96-, 90-, 64-, and 55-kd proteins was detected by immunoblotting using 2 phospho-Akt substrate antibodies and a phosphoprotein kinase A substrate antibody. In addition, dephosphorylation of a 105-kd protein was detected using a phospho-ATM/ATR substrate antibody. In contrast, no dephosphorylation was observed using a phosphoprotein kinase C substrate antibody, and increased tyrosine phosphorylation of 32- and 20-kd proteins was detected in C compared with N sperm. Immunolocalization experiments revealed subtle changes in the pattern expression as well as a reduction of phosphorylation in C sperm. Whereas sperm incubated in N medium containing dibutyryl cAMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX) did not show protein dephosphorylation, incubation in C medium with dbcAMP/IBMX showed dephosphorylation as well as increased phosphorylation of other proteins (p68, p51, and p29). Finally, calyculin A, a phosphatase inhibitor, prevented dephosphorylation of p96, p90, p64, and p55 but not p105. Based on these data, we propose 2 pathways of protein dephosphorylation that are active during capacitation and independent of cAMP. Together, this provides direct evidence for more complex S/T phosphorylation dynamics than has been previously described for sperm undergoing capacitation.

Key words: S/T, signaling, phosphorylation, mammalian, capacitation.

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Spermatozoa must undergo a series of events in the female tract that enables them to fertilize an oocyte; collectively these events are known as capacitation. Discovered in the early 1950s (Austin, 1951; Chang, 1951), capacitation is believed to facilitate the development of hyperactivated motility, zona binding, and the acrosome reaction. Capacitation can also be induced in vitro by incubation of the sperm in specially defined media (Salicioni et al, 2007; Gadella et al, 2008).

Although there are variations in the media used for in vitro capacitation, most contain bicarbonate, calcium, and bovine serum albumin (BSA; Visconti and Kopf, 1998). Bicarbonate is particularly important for inducing capacitation in mammalian sperm in general and particularly in the boar (Okamura et al, 1985; Harrison, 1996; Flesch and Gadella, 2000). Bicarbonate activates an adenylyl cyclase known as SACY that is abundant in sperm and acts as a bicarbonate sensor to generate cAMP (Okamura et al, 1985; Chen et al, 2000). The subsequent activation of protein kinase A (PKA; Visconti et al, 1995; Flesch and Gadella, 2000) induces, via an unknown signaling mechanism, tyrosine phosphorylation of several proteins in boars (Kalab et al, 1998; Flesch et al, 1999; Harayama, 2003), mice (Visconti et al, 1995), bulls (Galantino-Homer et al, 1997), and humans (Leclerc et al, 1996). In addition, in vitro capacitation may be regulated by BSA probably because of its ability to serve as a sink for the removal of cholesterol from the sperm plasma membrane which causes membrane fluidity changes (Visconti et al, 1999a,b).

Protein phosphorylation is known to regulate sperm motility involving tail proteins, and this has been demonstrated in the boar (Holt and Harrison, 2002). It may also be important for zona pellucida recognition involving head proteins at fertilization (Salicioni et al, 2007; Boerke et al, 2008). Although sperm phosphoproteins have been elucidated in many species, their roles and regulation during capacitation are still not fully understood. The involvement of PKA has been demonstrated; inhibitors of PKA activity are able to inhibit

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tyrosine phosphorylation as well as capacitation (Visconti et al, 1995; Leclerc et al, 1996). It has been hypothesized that PKA phosphorylates some enzymes/proteins as an intermediate step and that these phosphorylated PKA substrates are involved in capacitation-related protein tyrosine phosphorylation and its regulation (Visconti et al, 1995; Aitken et al, 1998; Visconti et al, 2002).

Many studies have investigated tyrosine phosphorylation in mammalian sperm (Salicioni et al, 2007), even though it is far less frequent in vertebrate somatic cells than serine/threonine (S/T) protein phosphorylation (1:1800:2000 for tyrosine:S:T phosphorylation events; Hunter, 1998). In contrast, there have been very few studies on S/T protein phosphorylation in sperm, and our understanding is therefore very limited. One major reason for this inequality is the general availability of antibodies that recognize phosphorylated tyrosine residues, whereas good-quality antibodies that specifically recognize phospho-(S/T) proteins/peptides have only become available in recent years. These new reagents are typically antibodies generated against the consensus sequences of individual kinases (eg, the PKB/Akt pathway) and represent useful reagents to survey the dynamics of S/T phosphorylation (Alessi et al, 1996).

The aim of this study was to investigate changes in phospho-(S/T) proteins in boar sperm incubated under capacitating (C) conditions using immunoblotting and immunofluorescence. We used 5 different phospho-(S/T) kinase substrate antibodies and detected dephosphorylation of 5 proteins. This is the first time it has been shown that multiple dephosphorylation events occur in a bicarbonate-dependent fashion in mammalian sperm. Moreover, studies with dibutyryl (dbcAMP)/3-isobutyl-1-methylxanthine (IBMX) and a phosphatase inhibitor suggest that dephosphorylation occurs through 2 pathways independent of cAMP. Together, these studies suggest that protein dephosphorylation may play a role in those events leading to sperm capacitation.

Materials and Methods

Sperm Preparation

Boar semen ejaculates were collected from Landrace boars and supplied in extender buffer (JSR Healthbred, Southburn, Driffield, Yorkshire, United Kingdom). Spermatozoa were isolated by centrifugation (750 × *g* for 20 minutes with brake off) through a 2-step discontinuous gradient of 35% and 70% isotonic Percoll (15 mL of each gradient overlaid with 15 mL of semen in extender buffer; Sigma-Aldrich Co Ltd, Poole, United Kingdom). Isotonic Percoll was prepared by first mixing Percoll with 10 × M medium (1.37 M NaCl, 25 mM KCl, 200 mM HEPES, 100 mM glucose, pH 7.55) (12:1 ratio) and then checking the osmolality confirm it was 285 to 300 mOsm/kg. This mixture was diluted to the appropriate concentration with 1 × M medium. After removal of the Percoll layers, the resultant loose pellet was resuspended in Tris-buffered sucrose solution (5 mM Tris, 0.25 M sucrose, pH 7.4; 10 mL) and subjected to further centrifugation (500 × *g* for 5 minutes). The pelleted cells were then resuspended in the appropriate buffer for incubation.

Sperm Incubation

Cells prepared as described were incubated under either noncapacitating (N) or C conditions at a concentration of 10 million/mL in 10 mL of media. For C conditions, Tyrode medium (100 mM NaCl, 21.7 mM lactate, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 15 mM $NaHCO_3$, 100 μg/mL kanamycin; 290–300 mOsm/kg, pH 7.4) containing 0.3% (wt/vol) BSA (Fraction V; Cat 05477, Sigma-Aldrich) was used with the cells in a tube with the screw cap loose at 38.5°C in a humidified incubator with 5% CO_2 in air (Parrish et al, 1988; Gadella and Harrison, 2000). N conditions were the same except that the Tyrode medium was prepared without $NaHCO_3$ (but with an additional 16 mM NaCl to maintain the same osmolality) and samples were incubated in airtight sealed tubes. Typically, samples were recovered for blotting and immunofluorescence experiments following a 1-hour incubation. In addition, the blotting data presented in Figures 1 through 3 also show samples recovered after 15 and 30 minutes. Finally, immunofluorescence data in Figure 1 following a 3-hour incubation are also presented.

In experiments in which the influence of cAMP on S/T protein phosphorylation was evaluated, sperm were incubated for 1 hour under N or C conditions in the absence or presence of 1 mM dbcAMP (Sigma-Aldrich), which is a cAMP analog, and 100 μM IBMX (Sigma-Aldrich), which is a phosphodiesterase inhibitor. In experiments in which the role of phosphatases on S/T protein phosphorylation was investigated, cells were incubated for 1 hour under N or C conditions in the absence or presence of either 100 or 250 nM calyculin A (Sigma-Aldrich), which is a pharmacologic phosphatase inhibitor of protein phosphatase 2A (PP2A) and PP1 (IC_{50}, 0.5–1.0 nM and 2.0 nM, respectively; Ishihara et al, 1989).

Assessment of Sperm Motility, Viability, and Acrosomal Integrity

Sperm motility was assessed by subjective observation. The motility was observed in a 20-μL aliquot of sperm suspension on a slide under a bright-field microscope. Sperm viability was assessed using a LIVE/DEAD sperm viability kit (Invitrogen Ltd, Paisley, United Kingdom). SYBR14 dye (5 μL; 100 nM final concentration) was added to 1 × 10^6 cells in 1 mL N or C medium following a 1- or 2-hour incubation and the mixture further incubated for 10 minutes at 38.5°C. Propidium iodide (5 μL; 12 μM final concentration) was then added, and the cells were incubated for a further 10 minutes at 38.5°C. Sperm were evaluated using a Leica fluorescence microscope, and 200 cells were counted for each treatment. Acrosomal status was

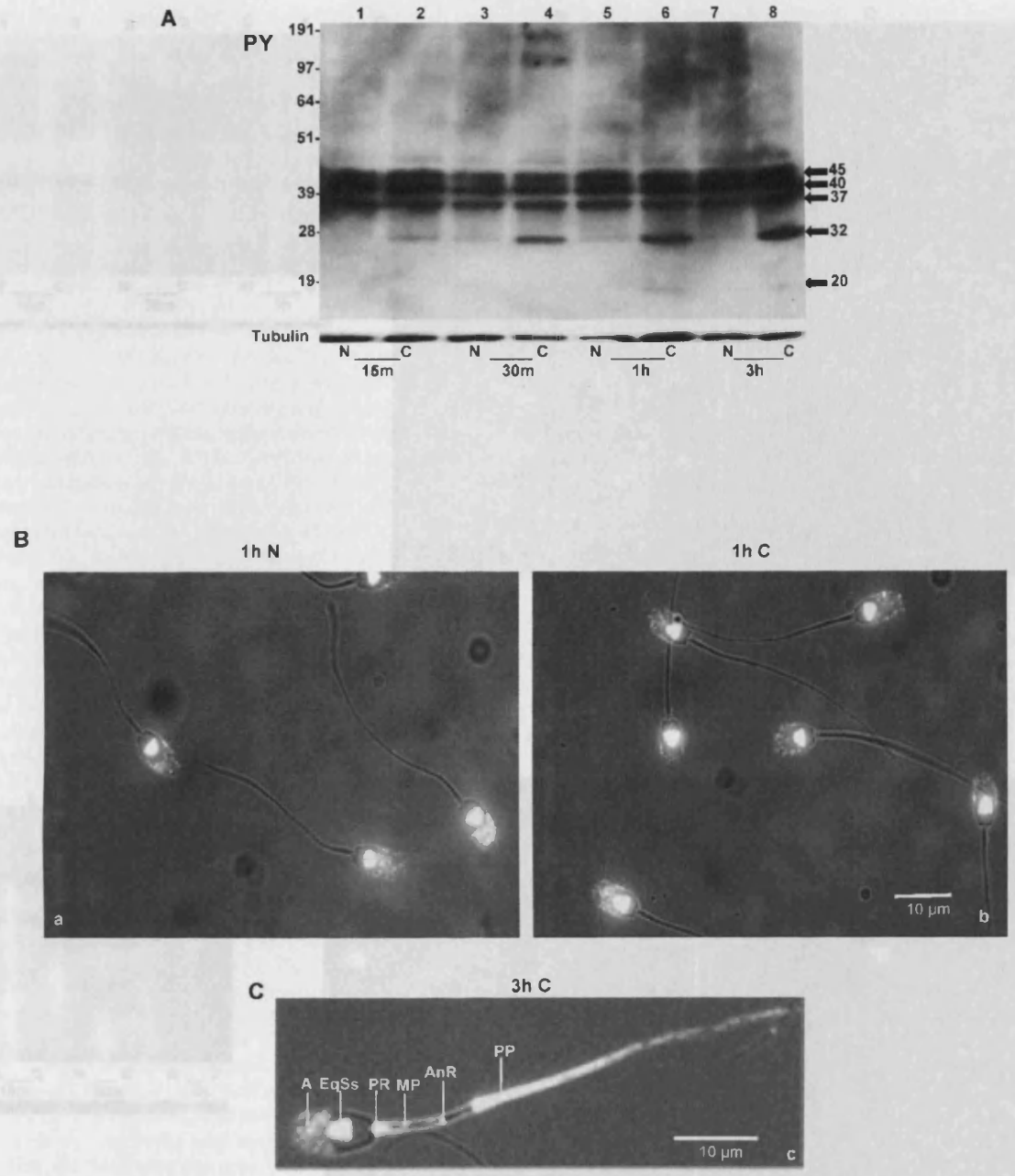
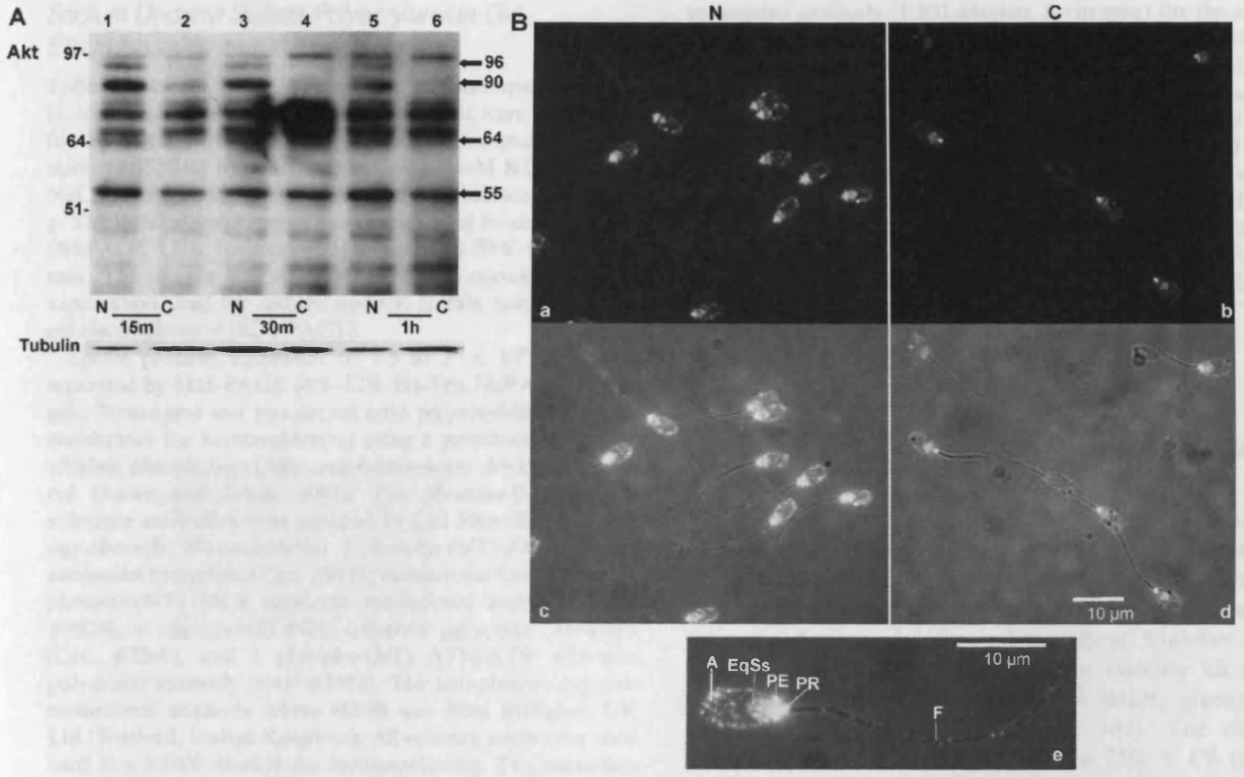


Figure 1. Protein tyrosine phosphorylation in sperm incubated under capacitating (C) conditions is similar as that in previously reported studies. **(A)** Immunoblot showing proteins detected with anti-phosphotyrosine antibody for sperm incubated under noncapacitating (N) and C conditions for 15 minutes, 30 minutes, 1 hour, and 3 hours. **(B)** Immunolocalization of phosphoproteins detected with the same antibody as in Panel A in fixed sperm cells after a 1-hour incubation under N or C conditions. **(C)** Immunolocalization of phosphoproteins detected with the same antibody as in Panel A in fixed sperm cells after a 3-hour incubation under C conditions. The results shown are representative of at least 10 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; EqSs, equatorial subsegment; PR, posterior ring; MP, midpiece; ApR, apical ridge; PP, principal piece.

assessed by indirect immunofluorescence using a monoclonal antibody (designated as 18.6) as described previously (Brewis et al, 1996). Briefly, 18.6 recognizes a sperm epitope within the acrosomal content, and fixed cells displaying even fluorescence in the acrosomal region are scored as acrosome intact. Nuclear staining was also performed using 4,6 dimidine-2-phenylindole dihydrochloride (Invitrogen) added to the secondary antibodies (0.2 µL/100 mL).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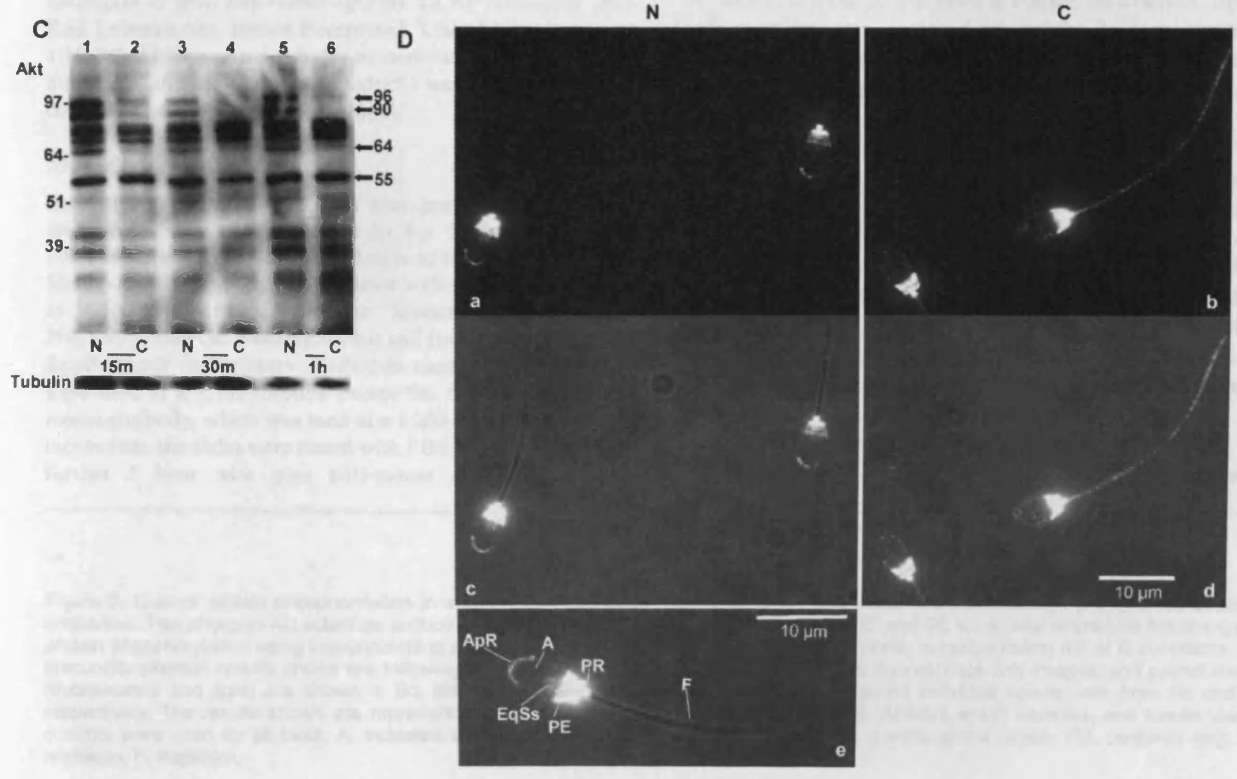
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Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

Following incubation in either N or C medium, sperm aliquots (1×10^7 cells) were collected. These aliquots were centrifuged for 10 minutes at 5000 × *g*, washed in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), and again centrifuged for 10 minutes at 5000 × *g*. The isolated sperm pellet was resuspended in sample buffer (NuPAGE LDS; Invitrogen) and heated at 70°C for 10 minutes. The sample was recentrifuged for 5 minutes, and the supernatant used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Sperm proteins equivalent to 1.5 to 2 × 10^6 cells were separated by SDS-PAGE (4%–12% Bis-Tris NuPAGE Novex gels; Invitrogen) and transferred onto polyvinylidene fluoride membranes for immunoblotting using a previously described alkaline phosphatase (AP) chemiluminescent detection protocol (Rowe and Jones, 2001). The phospho-(S/T) kinase substrate antibodies were supplied by Cell Signaling Technology (Beverly, Massachusetts): 2 phospho-(S/T) Akt substrate antibodies (polyclonal Cat. #9611; monoclonal Cat. #9614), a phospho-(S/T) PKA substrate monoclonal antibody (Cat. #9624), a phospho-(S) PKC substrate polyclonal antibody (Cat. #2261), and a phospho-(S/T) ATM/ATR substrate polyclonal antibody (Cat. #2851). The anti-phosphotyrosine monoclonal antibody (clone 4G10) was from Millipore UK Ltd (Watford, United Kingdom). All primary antibodies were used at a 1:1000 dilution for immunoblotting. The secondary antibodies used were goat anti-mouse IgG (H+L) AP conjugate or goat anti-rabbit IgG (H+L) AP conjugate (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) at a 1:10 000 dilution. An α-tubulin monoclonal antibody (1:1000 dilution; Cat. #T9026; Sigma-Aldrich) was used as a loading control for each blot (50-kd epitope).

Indirect Immunofluorescence

Incubated sperm cells (1×10^5) were gently smeared onto a microscopic slide and allowed to air dry. Slides were fixed in 100% methanol for 5 minutes and allowed to air dry for 1 hour. Slides were then incubated for 1 hour with different antibodies at 38.5°C in a wet box. Please see "Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting," for details about the primary antibodies used and note that all were used at a 1:100 dilution except for the anti-phosphotyrosine antibody, which was used at a 1:200 dilution. Following incubation, the slides were rinsed with PBS and incubated for a further 1 hour with goat anti-mouse Alexa Fluor 488–conjugated antibody (1:300 dilution; Invitrogen) for the anti-phosphotyrosine antibody and with goat anti-rabbit Alexa Fluor 488–conjugated antibody (1:300 dilution; Invitrogen) for phospho-(S/T) kinase substrate antibodies. For each primary antibody used, a control slide was processed and analyzed for which the primary antibody incubation was excluded and the cells were just incubated with secondary antibody. Slides were washed with PBS and mounted with Slow Fade Light antifade solution (Dako UK Ltd, Ely, United Kingdom). Slides were assessed by epifluorescence ultraviolet microscopy at a 492-nm wavelength with ×40 oil objective lens magnification to determine localization of detected phosphoproteins.

Results

Protein Tyrosine Phosphorylation Is Increased in Sperm Incubated Under C Conditions

The first step of this study was to investigate tyrosine phosphorylation in sperm incubated under experimental C conditions. To this end, we generated protein extracts and prepared slides of fixed sperm cells and in parallel determined sperm viability and movement. Viability was assessed using a LIVE/DEAD sperm viability kit and classified as live (plasma membrane intact; green) or dead (plasma membrane damaged; red). The mean (±SEM) percentage of live sperm was 75% ± 1% (n = 4) after a 1-hour incubation under C conditions and in N cells was 81% ± 3% after a 1-hour incubation. Sperm cell motility was assessed by bright-field microscopy, and the majority of sperm incubated in C medium showed rapid progressive motility after a 5-minute incubation. The motility increased over time and reached a plateau after 2 hours of incubation, with a similar pattern after 3 hours. In contrast to C sperm, N sperm cells showed no movement through the microscopic field in all experiments but instead demonstrated a vibrational movement ("static vibrance") caused by sperm sticking to the microscope slide owing to a lower lateral head displacement than that of the C cells. This is consistent with observations by other researchers using the pig as a model system (Gadella, personal communication).

With confidence that we had viable sperm which was motile under C conditions, whole-cell lysates were

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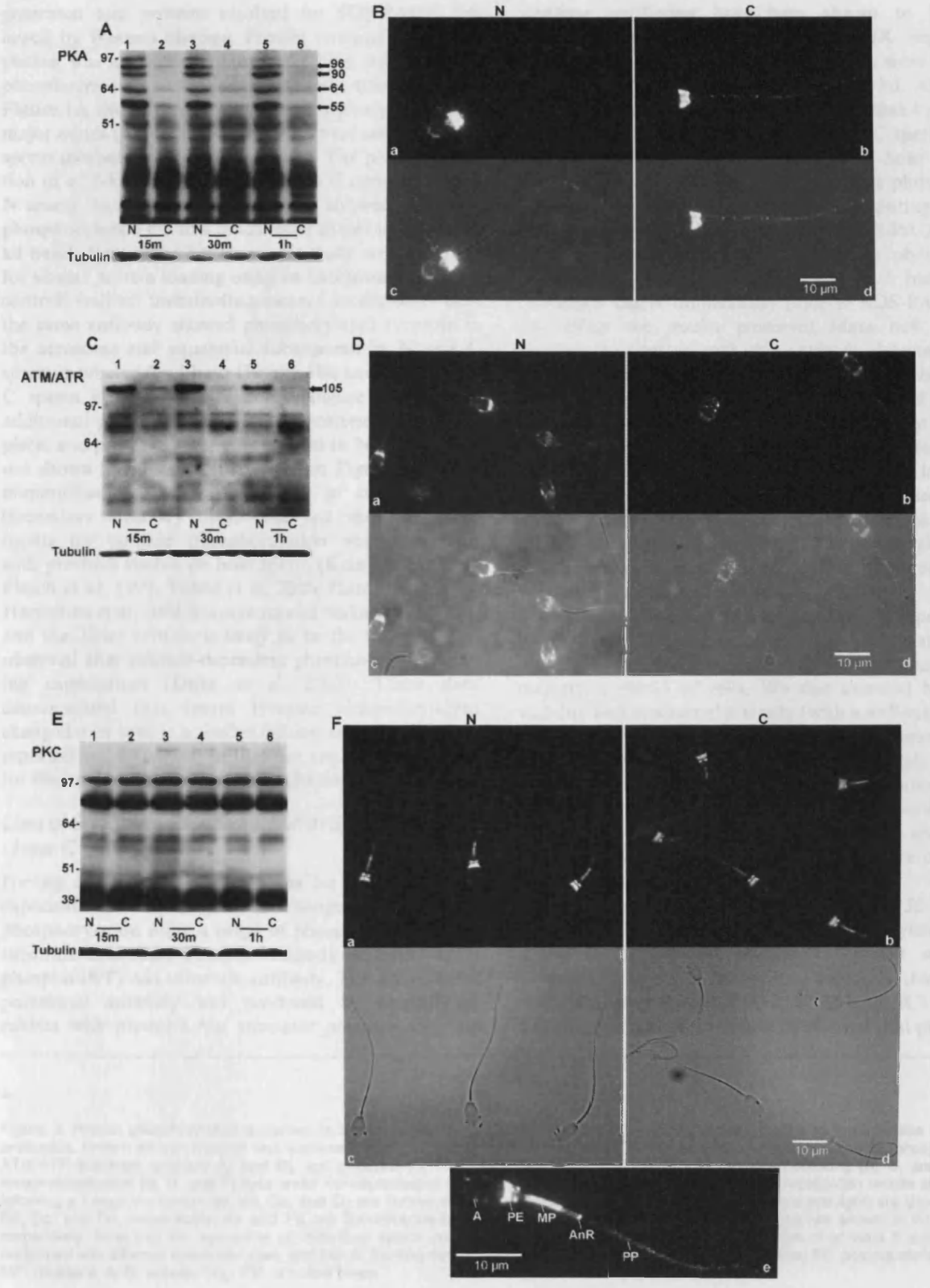
Figure 2. Loss of protein phosphorylation in sperm incubated under capacitating (C) conditions was detected with phospho-Akt substrate antibodies. Two phospho-Akt substrate antibodies, a polyclonal **(A and B)** and a monoclonal **(C and D)**, were used to analyze the changes in protein phosphorylation using immunoblotting **(A and C)** and immunolocalization **(B and D)** under noncapacitating (N) or C conditions. The immunolocalization results shown are following a 1-hour incubation. Ba, Bb, Da, and Db are fluorescence-only images, and paired images (fluorescence and light) are shown in Bc, Bd, Dc, and Dd, respectively. Be and De represent individual sperm cells from Bc and Dc, respectively. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A, indicates acrosome; EqSs, equatorial subsegment; PE, postequatorial region; PR, posterior ring; MP, midpiece; F, flagellum.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PKA
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generated and proteins resolved by SDS-PAGE followed by Western blotting. Protein tyrosine phosphorylation was assessed by immunoblotting with an anti-phosphotyrosine monoclonal antibody (clone 4G10). Figure 1A shows protein tyrosine phosphorylation of 3 major bands (45, 40, and 37 kd) that were unchanged in sperm incubated under C conditions. The phosphorylation of a 32-kd band was increased in C compared with N sperm. In addition, a 20-kd band showed increased phosphorylation but to a much lesser extent than the 32-kd band. Note that all blots in this study were assessed for similar protein loading using an anti-tubulin loading control. Indirect immunofluorescence localization with the same antibody showed phosphorylated tyrosine in the acrosome and equatorial subsegment in N and C sperm incubated for 1 hour (Figure 1Ba and b), whereas C sperm incubated for 3 hours (Figure 1C) showed additional phosphorylation in the posterior ring, midpiece, and principal piece (not present in N sperm; data not shown but the same pattern as in Figure 1Ba). No immunofluorescence was observed in control slides (secondary antibody only; data not shown). These results for tyrosine phosphorylation were consistent with previous studies on boar sperm (Kalab et al, 1998; Flesch et al, 1999; Tardif et al, 2001; Harayama, 2003; Harayama et al, 2004; Harayama and Nakamura, 2008), and the 32-kd protein is likely to be the same as that observed after calcium-dependent phosphorylation during capacitation (Dubé et al, 2003). These data demonstrated that sperm tyrosine phosphorylation changed over time in a similar fashion as that previously reported and convinced us that our system was suitable for the study of boar sperm capacitation in vitro.

Loss of S/T Protein Phosphorylation in Sperm Incubated Under C Conditions

Having established the conditions for in vitro sperm capacitation, we investigated the changes in S/T protein phosphorylation using a range of phospho-(S/T) kinase substrate antibodies. The first antibody we tested was a phospho-(S/T) Akt substrate antibody. This commercial polyclonal antibody was produced by immunizing rabbits with phospho-Akt substrate peptides, and the resulting antibodies have been shown to have a specificity of R/K-X-R/K-X-X-T*/S* (X represents any amino acid). The results in Figure 2A show distinct bands, particularly in proteins above 50 kd. Although some bands did not change, we observed that 4 proteins of 96, 90, 64, and 55 kd were reduced in C sperm when compared with N sperm throughout a 1-hour incubation (Figure 2A). It should be noted that phosphatase inhibitors were not routinely added during sperm washing and extraction prior to SDS-PAGE, but control experiments using a broad-range phosphatase inhibitor cocktail added to both the wash buffer and extraction buffer immediately prior to SDS-PAGE did not affect the results presented (data not shown). Immunolocalization with this antibody detected phospho-(S/T) protein in the flagellum (low levels), acrosome, equatorial subsegment, postequatorial region, and posterior ring in N sperm incubated for 1 hour (Figure 2Ba). Less phosphorylation was present in C sperm incubated for 1 hour (Figure 2Bb). No immunofluorescence was observed in control slides (secondary antibody only), and the same control was employed for all the immunofluorescence studies using phospho-(S/T) kinase substrate antibodies. In all cases, no fluorescence was observed (data not shown).

Acrosomal integrity was assessed in all experiments by observing the immunofluorescence micrographs, and we confirmed that the acrosomes were intact in the majority (>90%) of cells. We also assessed both cell viability and acrosomal integrity (with a well-established immunofluorescence assay using the 18.6 monoclonal antibody) before and after washing immediately prior to SDS-PAGE. At least 90% of cells were acrosome intact, and no differences were observed between washed and nonwashed cells and between N and C sperm. In addition, no differences in cell viability were observed (data not shown).

The 4 phosphoproteins of 96, 90, 64, and 55 kd were detected in N sperm but were dephosphorylated in C sperm by a different phospho-(S/T) Akt substrate antibody, which is a monoclonal antibody that recognizes the motif R-X-R-X-X-T*/S* (Figure 2C). Immunolocalization with this antibody showed that phospho-

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Figure 3. Protein phosphorylation dynamics in sperm incubated under capacitating (C) conditions using 3 other phosphokinase substrate antibodies. Protein phosphorylation was analyzed using a phospho-serine/threonine (S/T) PKA substrate antibody **(A and B)**, phospho-(S/T) ATM/ATR substrate antibody **(C and D)**, and phospho-(S) PKC substrate antibody motif **(E and F)**. Immunoblotting **(A, C, and E)** and immunolocalization **(B, D, and F)** data under noncapacitating (N) or C conditions are presented. The immunolocalization results shown are following a 1-hour incubation. Ba, Bb, Da, and Db are fluorescence-only images and paired images (fluorescence and light) are shown in Bc, Bd, Dc, and Dd, respectively. Fa and Fb are fluorescence-only images, and paired light microscopy images are shown in Fc and Fd, respectively. Note that Fe represents an individual sperm cell from Fa. The results shown are representative of at least 5 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; PE, postequatorial region; MP, midpiece, AnR, annular ring; PP, principal piece.

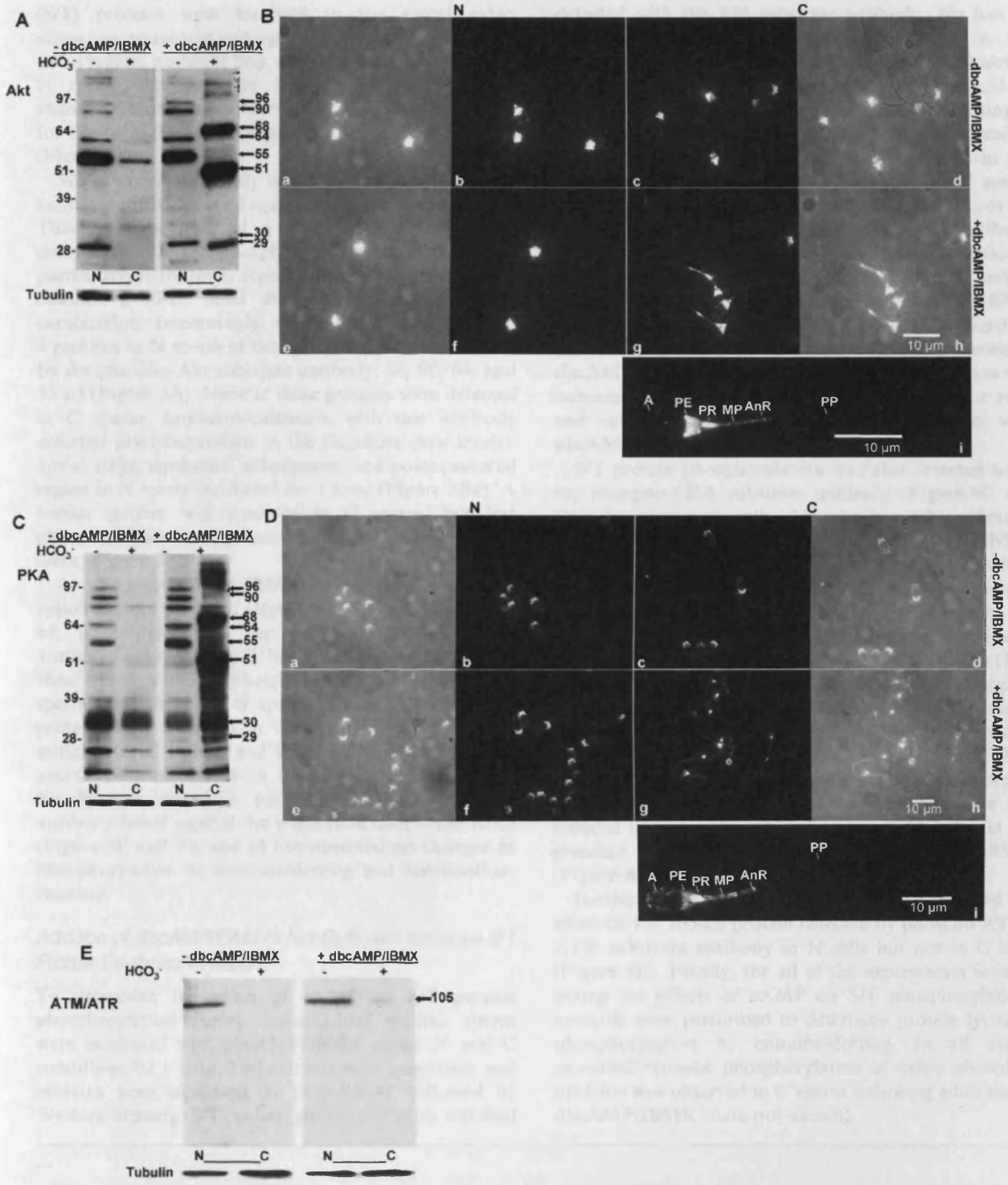

Figure 4. The effect of dibutyryl cAMP (dbcAMP)/3-isobutyl-1-methylxanthine (IBMX) on protein serine/threonine (S/T) phosphorylation in sperm incubated under capacitating (C) conditions. Cells were treated with dbcAMP/IBMX under both noncapacitating (N) and C conditions. Subsequently, they were analyzed by immunoblotting **(A, C, and E)** and immunolocalization **(B and D)**, as described. Protein phosphorylation was analyzed using a phospho-(S/T) Akt substrate polyclonal antibody **(A and B)**, a phospho-(S/T) PKA substrate antibody **(C and D)**, and a

(S/T) proteins were localized in the apical ridge, acrosome, equatorial subsegment (low levels), postequatorial region, posterior ring, and flagellum (low levels) in N sperm incubated for 1 hour (Figure 2Da). No phosphorylation was observed in C sperm incubated for 1 hour in the apical ridge and equatorial subsegment (Figure 2Db).

We extended our study using a phospho-(S/T) PKA substrate antibody raised against the motif R-R-X-S*/T*. This antibody detected a wider range of proteins compared with the phospho-Akt substrate antibody, particularly in the lower region of the gel. For example, it detected a 35-kd band that was unchanged during capacitation. Interestingly, this antibody also detected 4 proteins in N sperm of the same size as those detected by the phospho-Akt substrate antibody: 96, 90, 64, and 55 kd (Figure 3A). None of these proteins were detected in C sperm. Immunolocalization with this antibody detected phosphoproteins in the flagellum (low levels), apical ridge, equatorial subsegment, and postequatorial region in N sperm incubated for 1 hour (Figure 3Ba). A similar pattern was observed in C sperm, but less phosphorylation was present in the equatorial subsegment (Figure 3Bb).

A phospho-(S/T) ATM/ATR substrate antibody, raised against the motif (Hyd-S*/T*-Q), was investigated. In contrast with the previous antibodies, this antibody detected only 3 bands. Interestingly, one of these bands, a 105-kd phosphoprotein was absent in C sperm compared with N sperm (Figure 3C). Phosphoproteins were localized in the apical ridge, acrosome, postequatorial region, and flagellum (low levels) in N and C sperm incubated for 1 hour (Figure 3Da and b). Finally, we tested a phospho-(S) PKC substrate antibody raised against the motif (R/K-X-S*-Hyd-R/K) (Figure 3E and Fa and b) but observed no changes in phosphorylation by immunoblotting and immunofluorescence.

Addition of dbcAMP/IBMX Is Not Sufficient to Cause S/T Protein Dephosphorylation

To determine the effect of cAMP on S/T protein phosphorylation during capacitation, washed sperm were incubated with dbcAMP/IBMX under N and C conditions for 1 hour. Cell extracts were generated, and proteins were separated by SDS-PAGE followed by Western blotting. S/T protein phosphorylation was first detected with the Akt substrate antibody. No loss of phosphorylation was detected in N sperm in the presence of dbcAMP/IBMX (Figure 4A). As observed previously, the phosphoproteins of 96, 90, 64, and 55 kd were absent in C compared with N sperm. Interestingly, additional phosphoproteins (eg, bands were observed at 68, 51, and 29 kd) were observed in C sperm in the presence of dbcAMP/IBMX compared with C sperm incubated in the absence of dbcAMP/IBMX or N sperm. Phospho-(S/T) proteins detected by this antibody were localized in the acrosome (low levels), equatorial subsegment, and postequatorial region in N and C sperm incubated in the absence of dbcAMP/IBMX (Figure 4Ba and b). This antibody was localized in similar cellular regions in N cells in the presence of dbcAMP/IBMX (Figure 4Bc). The phosphorylation was enhanced in the postequatorial region, posterior ring, and midpiece following a 1-hour incubation with dbcAMP/IBMX in C sperm (Figure 4Bd).

S/T protein phosphorylation was also detected using the phospho-PKA substrate antibody (Figure 4C and D). As observed with the phospho-Akt substrate antibody, treatment of N sperm with dbcAMP/IBMX did not cause the loss of protein phosphorylation, which was observed in C sperm. Again, enhanced protein phosphorylation was observed in C sperm in the presence of dbcAMP/IBMX compared with C sperm incubated without dbcAMP/IBMX or N sperm (Figure 4C). Immunofluorescence analysis with phospho-PKA substrate antibody detected phosphoproteins localized in the postequatorial region in N sperm incubated in the presence or absence of dbcAMP/IBMX (Figure 4Da and c). Phosphorylation was enhanced in the posterior ring, midpiece, and principal piece but reduced in the postequatorial region in C sperm in the presence but not in the absence of dbcAMP/IBMX (Figure 4Db and d).

Incubation of sperm with dbcAMP/IBMX had no effect on the 105-kd protein detected by phospho-ATM/ATR substrate antibody in N cells but not in C cells (Figure 4E). Finally, for all of the experiments investigating the effects of cAMP on S/T phosphorylation, controls were performed to determine protein tyrosine phosphorylation by immunoblotting. In all cases, increased tyrosine phosphorylation of many phosphoproteins was observed in C sperm following addition of dbcAMP/IBMX (data not shown).

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phospho-(S) ATM/ATR substrate antibody (**E**). Bb, Bc, Bf, Bg, Db, Dc, Df, and Dg are fluorescence-only images, and paired images (fluorescence and light) are shown in Ba, Bd, Be, Bh, Da, Dd, De, and Dh, respectively. Bi and Di represent individual sperm cells from Bg and Dg, respectively. The results shown are representative of at least 3 experiments performed with different sperm samples, and tubulin loading controls were used for all blots. A indicates acrosome; PE, postequatorial region; PR, posterior ring; MP, midpiece; AnR, annular ring; PP, principal piece.

Calyculin A Inhibits Most Protein Dephosphorylation Observed in Sperm Incubated Under C Conditions

The final step in this study was to investigate the effect of calyculin A, which is a PP2A and PP1 phosphatase inhibitor, to assess whether protein dephosphorylation was caused by these classes of phosphatase. Sperm were incubated in the presence or absence of calyculin A at concentrations of 100 and 250 nM under N or C conditions. No obvious differences in sperm motility in C sperm were observed. Protein extracts were generated and processed as before. Calyculin A prevented the loss of detection of 4 bands detected with the phospho-PKA substrate antibody in C sperm: p96, p90, p64, and p55 (Figure 5B). It also prevented the loss of similar bands detected with the Akt substrate polyclonal antibodies (Figure 5C) and Akt substrate monoclonal antibody (data not shown). However, calyculin A did not prevent the loss of the 105-kd protein detected by phospho-ATM/ATR substrate antibody in N cells but not in C cells (Figure 5A). This suggests 2 distinct mechanisms of protein dephosphorylation in boar sperm incubated under C conditions.

Discussion

This project was initiated to investigate S/T phosphorylation in sperm incubated under C conditions. We chose phosphokinase substrate antibodies that have been used to investigate other signaling systems but have not been widely used to study mammalian sperm proteins. Using these reagents, we identified the loss of phosphorylation of 5 different molecular-weight proteins, 4 through a calyculin A–sensitive phosphatase and 1 through a calyculin A–independent pathway. These dephosphorylation events could not be caused by dbcAMP/IBMX treatment alone. Together, these data indicate 2 pathways of phosphatase activity that are activated in sperm incubated under C conditions downstream of the bicarbonate sensor in a cAMP-independent manner.

We deliberately chose to study S/T phosphorylation using 4 antibodies that have not been used previously in sperm, including substrate antibodies for Akt (2 antibodies), ATM/ATR, and PKC. With the Akt substrate antibodies, we observed dephosphorylation of 5 proteins in sperm incubated under capacitating conditions, whereas increased phosphorylation of other proteins was observed following treatment with dbcAMP/IBMX. These changes contrasted with the PKC substrate antibody, which detected similar proteins under all experimental conditions. We detected dephosphorylation with a PKA substrate antibody that we chose as a comparison because it had been used in other studies (Harrison, 2004; O'Flaherty et al, 2004; Harayama and Miyake, 2006; Harayama and Nakamura, 2008; Kaneto et al, 2008). One previous study reported decreased phosphorylation of a single 100 kd phospho-(S/T) protein during capacitation using a different anti-phosphoserine antibody in the hamster (Jha and Shivaji, 2002). However, the majority of studies have focused on increases in phosphorylation. Thus, this study is the first to document multiple dephosphorylation events that occur in a bicarbonate-dependent fashion. Together, this provides direct evidence for more complex S/T phosphorylation dynamics than is generally described for sperm undergoing capacitation.

There was an overlap with the bands detected with the Akt substrate antibodies and the PKA substrate antibodies, particularly under N conditions. Despite the overlap, when the Akt substrate antibodies and the PKA substrate antibody were compared using immunofluorescence, the pictures look subtly different, probably owing to the detection of other proteins by the antibodies. Although different motifs of S/T PKA substrate antibody were used in this study than previously used by others on boar sperm, the immunolocalization of these phosphoproteins was similar (Harayama, 2003; Adachi et al, 2008). Interestingly, phosphorylation seemed to be lost in the head region with the Akt substrate monoclonal antibody (Figure 2D), which may suggest a role in zona binding. There is a similarity between the motifs recognized by the Akt and PKA substrate antibodies: the R-R-X-S*/T* motif of the PKA substrate would be included within the R-X-R-X-X-S*/T* of the Akt substrate antibody. Given this, we would predict that p96, p90, and p55 contain a motif that is made up of both sequences, perhaps R-X-R-R-X-S*/T*. This is valuable information that we will aim to use to identify these proteins as the next step in our studies.

These experiments show that S/T dephosphorylation, across all 5 proteins studied, occurs within 15 minutes of incubation of sperm in C conditions. This rapid response indicates a direct role for the bicarbonate sensor in stimulating protein dephosphorylation. The rapid dephosphorylation is paralleled by previous reports that merocyanine-reported boar sperm membrane fluidization occurs within minutes after bicarbonate addition (Gadella and Harrison, 2000). However, treatment of sperm with dbcAMP/IBMX in N conditions did not result in dephosphorylation, demonstrating that elevation of cAMP alone was not sufficient to cause dephosphorylation. This further supports the observation that the bicarbonate sensor is a critical regulator of the changes that occur inside sperm cells undergoing capacitation.

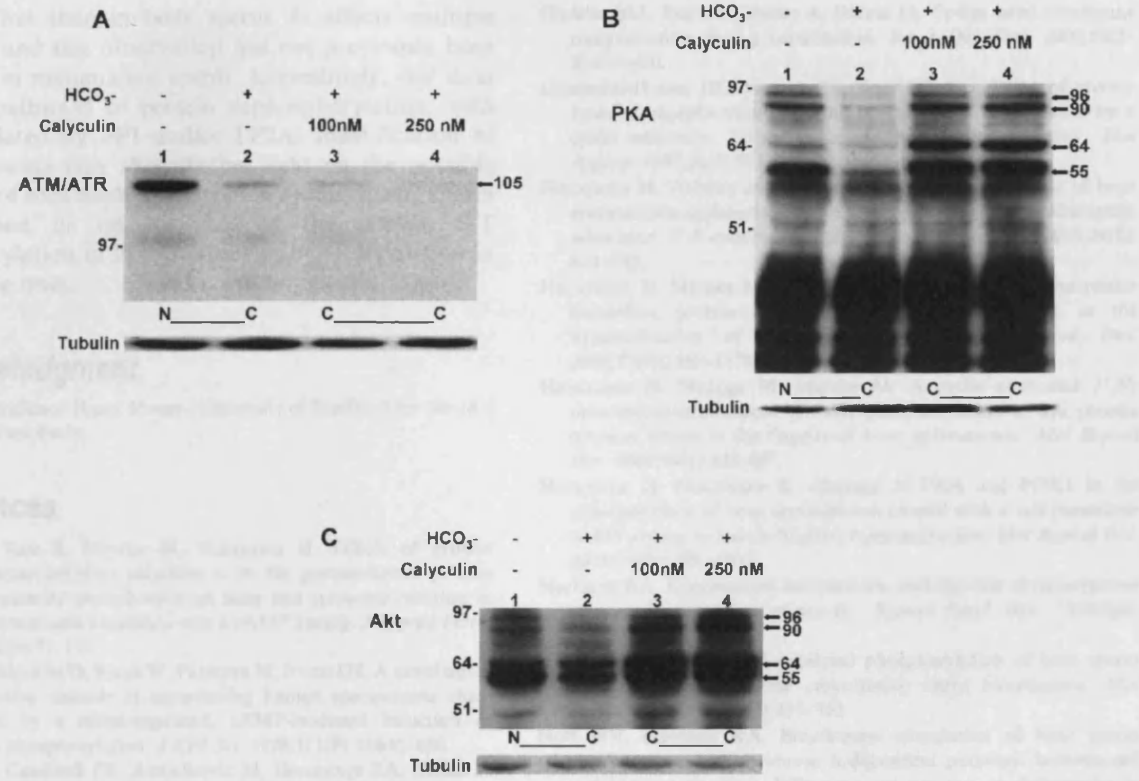

Figure 5. The effect of calyculin A on serine/threonine (S/T) dephosphorylation in sperm incubated under capacitating (C) conditions. Cells were treated with 2 concentrations of calyculin A, as indicated, under both noncapacitating (N) and C conditions. Subsequently, protein phosphorylation was analyzed by immunoblotting using a phospho-(S/T) ATM/ATR substrate antibody **(A)**, a phospho-(S/T) PKA substrate antibody **(B)**, and a phospho-(S/T) Akt substrate polyclonal antibody **(C)**. The results shown are representative of at least 3 experiments performed with different sperm samples, and tubulin loading controls were used for all blots.

One of the dephosphorylation events that was detected by the phospho-ATM/ATR substrate antibody was independent of the phosphatase inhibitor calyculin A, whereas dephosphorylation of the other proteins was sensitive to this compound. This suggests that 2 different pathways lead to protein S/T dephosphorylation: one involving either PP1 and/or PP2A and one that is independent. We investigated other inhibitors, including okadaic acid, but we could not demonstrate any dramatic changes in phosphorylation patterns. Calyculin A has previously been used as a phosphatase inhibitor in boar sperm, and it greatly enhanced the phosphorylation of S/T PKA proteins (Harrison, 2004) and caused increased sperm motility (Holt and Harrison, 2002). A recent study by Adachi et al (2008) using immunolocalization but not Western blotting reported S/T dephosphorylation in the postacrosomal region during capacitation. These proteins showed increased phosphorylation when sperm were incubated with calyculin A. It is possible that one of the bands observed in our study may also be the protein(s) observed by Adachi et al (2008), suggesting a possible functional role for these phosphorylated proteins before and after ejaculation in boar sperm.

Since dephosphorylation has been demonstrated as affecting multiple proteins in sperm incubated under C conditions, the next step is to identify these proteins. Unfortunately, the size of these proteins does not suggest any likely candidates based on the literature. Furthermore, although these are kinase substrate antibodies, it is possible that these proteins do not lie downstream of the kinases that denote the name of the antibody. This was shown to be true for the phospho-Akt substrate antibody, which also recognizes phosphorylation of S6 which is regulated by S6 kinase not Akt (Kane et al, 2002). Thus, we are currently undertaking a proteomic approach to characterize proteins of the appropriate molecular weight in sperm in which we will search for the R-X-R-R-X-S*/T* motif. In combination with immunoprecipitation, we believe this approach has the potential to identify these proteins from boar sperm.

In conclusion, S/T protein dephosphorylation in sperm incubated under C conditions has been reported

for the first time in boar sperm. It affects multiple proteins, and this observation has not previously been reported in mammalian sperm. Interestingly, our data show 2 pathways of protein dephosphorylation, with one regulated by PP1 and/or PP2A. Identification of these proteins may shed further light on the possible function of such dephosphorylation events during sperm capacitation or on the role of the protein S/T phosphorylation in N conditions prior to deposition in the female tract.

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